A *URA3* null mutant of *Candida albicans* (CAI-4) causes oro-oesophageal and gastric candidiasis and is lethal for gnotobiotic, transgenic mice (Tgε26) that are deficient in both natural killer and T cells

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Current data suggest that functional *URA3* genes are necessary for the full pathogenesis of *Candida albicans*. Herein it is shown that a putatively avirulent *URA3/URA3* null mutant of *C. albicans* (CAI-4) can colonize the murine alimentary tract, invade oro-oesophageal and gastric tissues with yeasts and hyphae, evoke a granulocyte-dominated inflammatory response, and kill transgenic mice that are deficient for both natural killer cells and T cells. Because *C. albicans*-colonized (gnotobiotic) mice lack a viable prokaryotic microbiota, this study also demonstrates that the gut microbiome is not required to supply the mutant's nutritional needs. The gnotobiotic murine model described herein can be used to assess the capacity of *C. albicans* mutants to colonize and infect cutaneous, mucosal and systemic tissues and kill the susceptible host via a clinically common, natural route of infection; namely the alimentary tract.

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INTRODUCTION

Candidiasis is increasing in an ever-expanding population of immunodeficient patients. *Candida* spp. now rank among the top four microbes isolated from clinical specimens (Pfaller & Diekema, 2007; Wisplinghoff *et al.*, 2003). Several decades ago, the increasing number of infections by *Candida* spp., many demonstrating resistance to antifungal therapy, stimulated major research efforts to study the molecular basis for their pathogenesis, identify their virulence genes, and improve the prophylaxis and therapy of these troublesome and at times lethal infections of immunodeficient patients. Because of the molecular revolution, *Candida albicans* 'has emerged as perhaps the best characterized fungal pathogen of humans in terms of analysis of gene function during infection' (Kronstad, 2007). Unfortunately, over the last several decades the number of patients with candidiasis has increased dramatically and resistance of *Candida* spp. to antifungal agents is associated with the increased lethality. Animal models of acute systemic candidiasis (ASC) are widely used to study the pathogenesis of *Candida*; however, ASC models provide no information on the capacity of *Candida* spp. to adhere to and colonize the alimentary tract and cause mucosal, skin or systemic infections of endogenous origin, which are the most common *Candida* infections observed in patients. Mucosal and systemic infections in patients are,

for the most part, mixed bacteria–*C. albicans* infections (Hermann *et al.*, 1999; Hogan & Kolter, 2002; Hogan *et al.*, 2004). The impact of the microbiome on gene expression by *C. albicans* *in vivo* and the host's immune responses (innate and acquired) are still largely unknown.

To date, molecular research efforts have neither identified the important virulence gene(s) that is activated by *C. albicans* during the most common natural route of infection (the alimentary tract) nor improved the prophylaxis and therapy needed to completely and permanently eliminate candidiasis as an infectious disease that is causing increasing deaths in susceptible patients (Pfaller, 1995; Pfaller & Diekema 2007).

One of the major breakthroughs in molecular biology research on *C. albicans* was the introduction of Ura-blaster constructs to carry out a targeted deletion of 'putative' virulence genes (Alani *et al.*, 1987; Fonzi & Irwin, 1993). The inactivated (deletion of orotidine-5'-monophosphate decarboxylase) *URA3* gene was used as a selectable marker to identify genes that play a role (putative) in the virulence of *C. albicans* even though it was known that purine and/or pyrimidine mutants of *C. albicans* were less pathogenic (ASC animal models) than prototrophic strains (Odds, 1979; Savage & Balish, 1971). Thus the *URA3* gene is not only involved in uridine biosynthesis but was also thought to be necessary for the full expression of systemic pathogenesis by *C. albicans* (Brand *et al.*, 2004; Lay *et al.*, 1998; Sundstrom *et al.*, 2002).

Abbreviations: ASC, acute systemic candidiasis; GF, germ-free.

Mutants made with Ura-blaster methodology presented several problems in identifying *Candida* virulence genes *in vivo* (Brand *et al.*, 2004; Lay *et al.*, 1998). Nutrients that can render purine or pyrimidine auxotrophs prototrophic are available *in vivo* (Manning *et al.*, 1984; Savage & Balish, 1971; Shepherd, 1985). Many Ura-blaster mutants of *C. albicans*, deleted of a putative virulence gene, were claimed to be avirulent, primarily on the basis of parenteral injections that produced acute systemic (pure culture) infections in immunocompetent mice (Brand *et al.*, 2004; Kirsch & Whitney, 1991; Lay *et al.*, 1998; Sundstrom *et al.*, 2002). The decreased pathogenesis of Ura-blaster mutants with disrupted virulence genes (putative) could not be attributed solely to a disruption of the targeted gene since the disrupted *URA3*, used as a selectable marker, interfered with the mutants' systemic pathogenicity. Random placement of *URA3* genes within the genome with Ura-blaster methodology further altered the mutants' systemic virulence and complicated attempts to reveal the true role of a targeted gene in the virulence of *C. albicans* (Brand *et al.*, 2004; Cheng *et al.*, 2003; Staab & Sundstrom, 2003; Sundstrom *et al.*, 2002). Of particular interest was the construction of *URA3* null mutants that were designated avirulent in animal models of ASC (Brand *et al.*, 2004; Kirsch & Whitney, 1991; Lay *et al.*, 1998; Shepherd, 1985). It was also reported that the null mutants were unable to colonize the oral cavity of xerostomic (hyposalivation) rats (Cole *et al.*, 1995). Thus *URA3* appeared to be necessary for the mucosal and systemic virulence of *C. albicans* (Lay *et al.*, 1998). An exception to the latter was demonstrated in a murine model, wherein it was shown that the deletion of *URA3* genes had little effect on corneal candidiasis (Jackson *et al.*, 2007).

Since pyrimidine biosynthesis appears to be important for the systemic virulence of *C. albicans* and very little is known about the role of *URA3* deletions in susceptibility to mucosal candidiasis and systemic candidiasis of endogenous (gut) origin, we assessed the capacity of a *URA3* null mutant of *C. albicans* (CAI-4) to colonize and infect oro-oesophageal and gastric tissues of gnotobiotic, transgenic mice that have combined defects in natural killer cells and T cells. Previously we demonstrated that these germ-free (GF) Tgε26 mice are susceptible to lethal oro-oesophageal candidiasis after they are colonized (monoassociated) with a pure culture of either a *URA3* homozygous (SC5314) or a heterozygous (CAF2-1) strain of *C. albicans* (Westwater *et al.*, 2007). Herein we report that a *URA3* null *C. albicans* mutant (CAI-4) colonized the murine alimentary tract, formed hyphae that invaded oro-oesophageal and gastric (non-secreting portion) tissues, and killed Tgε26 mice.

METHODS

Micro-organisms. *C. albicans* strains SC5314, CAF2-1 and CAI-4 were obtained from Dr Joseph Dolan (Nashville State Community College, Nashville, TN, USA). *C. albicans* SC5314 (*URA3/URA3*) is the wild-type parent of the genetically marked heterozygous CAF2-1

(*URA3/ura3*) and CAI-4, the *URA3* null mutant (*ura3/ura3Δ::imm434*) (Fonzi & Irwin, 1993). *C. albicans* strains were grown on Sabouraud dextrose agar or broth (SDA or SDB, respectively).

Mice. Transgenic epsilon 26 (Tgε26) mice were derived into the GF state at the University of Wisconsin Gnotobiotic Laboratory (Madison, WI, USA) and bred at the Medical University of South Carolina Gnotobiotic Facility (<http://www.musc.edu/gnotobiotic>). The Tgε26 mice were originally generated by overexpressing the full-length human CD3ε gene in C57BL/6xCBA/J mice and are defective in both natural killer cell and T-cell functions (Wang *et al.*, 1994). All animal procedures were approved by the Institutional Animal Care and Use Committee and followed the guidelines of the American Veterinary Medical Association.

Oro-oesophageal and gastric candidiasis. The alimentary tracts of GF Tgε26 mice were colonized with a pure culture of *C. albicans* by oral inoculation (Balish *et al.*, 2001). The cell inoculum was prepared from a 24 h culture (SDB at 37 °C), which had been washed twice in PBS, and counted with a haemocytometer. GF mice were provided with a drinking bottle containing a standardized *C. albicans* cell suspension (40 ml, 10^6 cells ml⁻¹ in PBS) for 4 h. The inoculum source was subsequently removed and the animals were given sterile drinking water for the remainder of the study. Microscopic examination and culturing of faecal pellets showed that the GF mice became heavily colonized with yeast and hyphal forms (*C. albicans*) within 24 h after oral inoculation. The mice remained chronically colonized with a pure culture of each wild-type or mutant strain for the duration of the 8-week study. The number of c.f.u. in the stomach contents, caeca and internal organs (kidney, spleen, liver) of euthanized mice was determined according to Balish *et al.* (2001). Data are presented as the number of viable *C. albicans* c.f.u. (mg dry wt)⁻¹ in tissue or alimentary tract contents.

Histopathology. To assess the presence of candidiasis, tissues were fixed, paraffin-embedded, sectioned (5 µm) and stained with periodic acid–Schiff reagent to visualize fungi, and counterstained with haematoxylin for characterization of host cells (Balish *et al.*, 2005). Three sections for each tissue harvested from at least three mice were ranked (blinded) for candidiasis as follows: 0, no hyphal penetration of mucosal surfaces per high-power field (HPF; ×400); 1+, 1–10 organisms per HPF; 2+, 10–50 organisms per HPF; 3+, abundant yeast and hyphae but infection is not confluent (50–100 organisms per HPF); and 4+, confluent invasion of mucosal surfaces with yeast and hyphae (>100 organisms per HPF). Tissue sections were also stained with haematoxylin and eosin to identify leukocytes infiltrating infected oro-oesophageal and gastric tissues.

Statistical analysis. All data were subjected to statistical analysis using Sigma Stat version 2.0 (SPSS Science). *P*-values were calculated by the Student's *t*-test and the Mann–Whitney Rank Sum test. *P*-values of <0.05 were considered significant. Survival after oral colonization with a pure culture of each of the three *C. albicans* strains was assessed with the Kaplan–Meier survival test.

RESULTS

Alimentary tract colonization

After oral inoculation, the *URA3* null mutant of *C. albicans* (CAI-4) colonized (chronically) the alimentary tract of the Tgε26 mice. Cultures of specimens from the stomachs and the caeca, over the 55-day experiment, demonstrated that viable *C. albicans* [6–8 log₁₀ (g contents)⁻¹] was present in

all specimens. These numbers were comparable to viable *C. albicans* isolated from Tgε26 mice colonized with *URA3*-positive (SC5314 or CAF2-1) strains of *C. albicans* (Westwater *et al.*, 2007).

Oro-oesophageal and gastric infections

All (Table 1) of the oesophagi and stomachs (cardia-antrum section) were infected in CAI-4 colonized mice; 70% also had lingual candidiasis. Thus the *URA3* null mutant (CAI-4) retains the capacity to colonize the alimentary tract and infect oro-oesophageal, gastric and lingual tissues with yeasts and hyphae.

Invasiveness (histopathology)

The severity of candidiasis (tissue invasiveness) in the oro-oesophageal and gastric tissues was ranked using histopathology scores of formalin-fixed and stained (periodic acid–Schiff and haematoxylin and eosin stains) infected tissues as described in Methods. CAI-4 candidiasis in the gastric tissue (non-secreting tissues) was ranked at 3.7. Although less severe, oesophageal tissues had a histopathology score of 2.5 and lingual tissue had a histopathology score of 1.4. The tissue most susceptible to CAI-4 was the non-secreting section of the murine stomach (Table 1; Fig. 1). These scores were similar to the severity scores that we observed with *URA3*-positive strains SC5314 and CAF2-1 (Balish *et al.*, 2001, 2005; Westwater *et al.*, 2007). Also no invasive candidiasis was evident in intestinal tract

Table 1. Oro-oesophageal and gastric colonization, infectivity and invasiveness of a *URA3* null mutant (CAI-4) of *C. albicans* for Tgε26 mice 3–8 weeks after colonization

	Result
Colonization*	
Stomach	6.5–7.8
Caecum	6.7–7.2
Infection†	
Stomach	8/8
Oesophagus	7/7
Tongue	5/7
Invasiveness‡	
Stomach	3.7
Oesophagus	2.5
Tongue	1.4

*Range ($\log_{10}$) of viable counts from stomachs and caeca of eight mice euthanized for histopathology at 3–8 weeks after colonization with CAI-4.

†Number of tissues infected/number of tissues examined from mice euthanized at 3–8 weeks after oral challenge.

‡Invasiveness was estimated by histopathology scores of candidiasis in tissues harvested at 3–8 weeks after oral challenge with CAI-4. 0, No infected areas seen; 4, confluent tissue invasion with yeasts and hyphae. See Methods for details.

Fig. 1. Yeasts and hyphae of CAI-4 (*URA3* null mutant) infecting the stomach (a) and oesophagus (b) of gnotobiotic Tgε26 mice at 4 weeks after oral colonization. Abscesses, composed predominantly of polymorphonuclear leukocytes, were prevalent in all infected tissues (periodic acid–Schiff stains at $\times 200$ magnification).

tissues distal to the non-secreting section of the stomach with any of the wild-type or mutant strains.

All *C. albicans*-infected (CAI-4 as well as *URA3*-positive strains) oro-oesophageal and gastric tissues manifested hyperkeratosis and an inflammatory response that consisted primarily of polymorphonuclear leukocytes (Fig. 1).

Lethality

Previously, it was demonstrated that *URA3*-positive strains of *C. albicans* were lethal for the Tgε26 mice within 3–5 weeks after alimentary tract colonization (Westwater *et al.*, 2007). The median survival times (55-day experiment) were 35, 21 and 41 days, respectively, for SC5314, CAF2-1 and CAI-4 (Fig. 2). The *URA3*-positive colonized mice (SC5314 or CAF2-1) apparently died from oesophageal occlusion and not from systemic candidiasis of endogenous origin since no *C. albicans* could be isolated from their internal organs. The *URA3* heterozygous strain (CAF2-1) was significantly ($P < 0.05$) more lethal for the Tgε26 mice than the wild-type strain (SC5314). In this study, the *URA3* null mutant (CAI-4), although significantly less lethal ($P < 0.05$) than the *URA3* homozygous and heterozygous strains, did kill 60 % of the Tgε26 mice in a 55-day experiment (Fig. 2).

DISCUSSION

Ura-blaster methodology is currently being used to identify genes that play a role in the virulence of *C. albicans*. Deletion of *URA3* genes from *C. albicans* has been reported to cause significant deficits in the production of at least 14 proteins, interferes with iron metabolism (Brand *et al.*, 2004), and decreases the mutants' mucosal and systemic pathogenesis for rodents. *URA3* deletion also adversely affects the growth rate of *C. albicans* and its capacity to form hyphae and attach to epithelial cells *in vitro* (Brand *et al.*, 2004; Kirsch & Whitney, 1991; Lay *et al.*, 1998; Shepherd, 1985; Sundstrom *et al.*, 2002).

Care has to be used in proclaiming a *C. albicans* mutant to be avirulent. *Candida* spp. are unique microbes that can cause a variety of infections (mucosal, skin, systemic) in a susceptible host. Proclamations on the avirulence of a *C. albicans* mutant should be withheld until Koch's postulates have been validated in relevant animal models that are susceptible to a major spectrum of *C. albicans* infections

(mucosal, skin and systemic). It is very unlikely that *C. albicans* uses the same virulence genes to infect all anatomical sites in a susceptible host. Just as important, similar animal model(s) should be used to fulfil Koch's molecular postulates to assess restored virulence in reintegrant strains.

Very few attempts (Cole *et al.*, 1995) have been made to contrast the virulence of *URA3*-positive or *URA3* null mutants for mucosal (oro-oesophageal and gastric) tissues and to produce systemic candidiasis of endogenous (alimentary tract) origin. A *URA3* null mutant was reported to be unable to colonize the oral cavity of xerostomic (hyposalivation) rats (Cole *et al.*, 1995). The presence, absence or chromosomal placement of the disrupted *URA3* gene can alter the virulence of *C. albicans* and this has created problems in the identification and validation of true virulence genes of *C. albicans* that are deleted with Ura-blaster methodology (Brand *et al.*, 2004; Sundstrom *et al.*, 2002). Before a *C. albicans* mutant is designated avirulent (mission accomplished?), animal models that characterize the effect of the deleted gene on mucosal, systemic and cutaneous infections should be used. The identification of true virulence genes of *Candida* is complicated by auxotrophs that have access to nutrients from the microbiota, the host or the diet that could render them prototrophic in the alimentary tract.

I am unaware of any studies with mutants of *C. albicans* that have fulfilled Koch's postulates for mutants and Koch's molecular postulates for reintegrant strains using animal models that mimic the spectrum of infections that *C. albicans* can cause in a susceptible host. P. Sundstrom and her colleagues have assessed the pathogenesis of *hwp1/hwp1* mutants in murine models of ACS, oro-oesophageal and gastric candidiasis and systemic candidiasis of endogenous origin (Staab & Sundstrom, 2003; Sundstrom *et al.*, 2002). The *hwp1/hwp1* null mutants were less virulent than wild-type or heterozygous strains in the murine models tested and the mutant was unable to cause systemic candidiasis of endogenous (alimentary tract) origin (Staab & Sundstrom, 2003; Sundstrom *et al.*, 2002; Balish *et al.*, 2005).

C. albicans is a natural biotin (vitamin H) auxotroph (Littman & Miwatani, 1963; Odds, 1979; Yamaguchi, 1974). Wild-type *C. albicans*, naturally auxotrophic for biotin and perhaps for other water-soluble vitamins (Odds, 1979), survives *in vivo*, forms hyphae, invades tissues and can kill a susceptible host. Perhaps the vitamin requirements of *C. albicans* can explain its unique (among fungi) requirement to survive, in nature, on mucosal surfaces. *C. albicans* has a natural vitamin H deficiency that also affects its polymorphism *in vitro* (Yamaguchi, 1974). Domergue *et al.* (2005) recently demonstrated how a natural vitamin deficiency affects the pathogenesis of *Candida glabrata*. It is very likely that all *C. albicans* mutants made with Ura-blaster methodology are also auxotrophic for biotin and perhaps for other water-soluble vitamins (Odds, 1979).

Fig. 2. Lethality of wild-type (SC5314) and *URA3* mutants (CAI-4, CAF2-1) of *C. albicans* for colonized gnotobiotic Tgε26 mice.

Combined biotin and URA3 defects could affect the pathogenesis of *C. albicans* and could explain the differences that we observed in lethality with URA3 homozygous and heterozygous strains.

Our experiments also demonstrated that CAI-4, CAF2-1 and SC5314 flourished in but were not able to infect (hyphae) sections of the alimentary tract distal to the non-secretory portion of the stomach. We also found no candidiasis of the skin and vagina. The *in vivo* environment (pH, oxidation–reduction potential) and availability of uridine, biotin and possibly other nutrients required by mutant and wild-type strains may vary at different anatomical sites, and differences in nutrient concentrations could explain why *C. albicans* was able to colonize but not infect some tissues (intestinal, vaginal, systemic organs and skin) in this otherwise susceptible gnotobiotic animal model.

Previous studies demonstrated that the *URA3* gene is important for *C. albicans* to adhere to epithelial cells in gnotobiotic (pure culture) studies *in vitro* (Bain *et al.*, 2001). Herein we demonstrate that *URA3* genes were apparently not necessary for *C. albicans* to adhere to and colonize the alimentary tract, form hyphae, infect oro-oesophageal and gastric tissues and kill the mice. Once again, this study demonstrates dramatic differences in the capacity of *C. albicans* strains to adhere to, form hyphae and invade tissues in gnotobiotic animal models versus studies carried out with tissue cultures *in vitro* (Bain *et al.*, 2001; Balish *et al.*, 2005; Lo *et al.*, 1997; Riggle *et al.*, 1999; Westwater *et al.*, 2007). Recent studies have demonstrated that several *C. albicans* mutants that could not form hyphae or infect tissue culture cells *in vitro* (Lo *et al.*, 1997) were fully capable of forming hyphae and invading tissues and killing the host under gnotobiotic conditions *in vivo* (Balish *et al.*, 2005; Bendel *et al.*, 2003; Riggle *et al.*, 1999). Obviously, gnotobiotic *in vivo* studies (animal models) are more realistic and applicable for studying the pathogenesis of *C. albicans* than ‘gnotobiotic’ *in vitro* studies with cultured cell lines.

In vivo tissue adherence, and the lack of adherence to cells *in vitro*, suggests that genes directing the formation of products important for *C. albicans* to adhere to, colonize and invade tissues may only be activated *in vivo*.

The gnotobiotic model used herein is unique and versatile in that it facilitates studies designed to assess the capacity of wild-type, mutant or reintegrant strains of *C. albicans* to produce several clinically relevant forms of candidiasis in one experimental animal model. The colonization and infectivity data derived from susceptible gnotobiotic animal models (immunocompetent or with defined immune defects) will make major contributions in identifying true virulence genes of *C. albicans* and in fulfilling Koch’s postulates and Koch’s molecular postulates for reintegrant strains.

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