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-blasters 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 candididiasis (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 candididiasis and systemic candididiasis 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 cell and T-cell functions (Wang et al., 1998). The decreased pathogenesis of Ura-blaster mutants 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.

**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::imm434) (Fonzi & Irwin, 1993). C. albicans strains were grown on Sabouraud dextrose agar or broth (SDA or SDB, respectively).

**Mice.** Transgenic epsilon 26 (Tg(26)) 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 Tg(26) 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 candididiasis.** The alimentary tracts of GF Tg(26) 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, 10⁶ 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 candididiasis, 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 candididiasis as follows: 0, no hyphal penetration; 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 Tg(26) 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 Tg:e26 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 haemotoxylin 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 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).

**Table 1. Oro-oesophageal and gastric colonization, infectivity and invasiveness of a URA3 null mutant (CAI-4) of C. albicans for Tg:e26 mice 3–8 weeks after colonization**

<table>
<thead>
<tr>
<th>Result</th>
<th>Stomach 6.5–7.8</th>
<th>Caecum 6.7–7.2</th>
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<tbody>
<tr>
<td><strong>Colonization</strong></td>
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<td>Caecum</td>
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<td>Tongue</td>
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<tr>
<td><strong>Invasiveness</strong></td>
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<tr>
<td>Stomach</td>
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<tr>
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<td>Tongue</td>
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*Range (log10) 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 Tg:e26 mice at 4 weeks after oral colonization. Abscesses, composed predominantly of polymorphonuclear leukocytes, were prevalent in all infected tissues (periodic acid–Schiff stains at ×200 magnification).
Lethality

Previously, it was demonstrated that URA3-positive strains of C. albicans were lethal for the Tg626 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 Tg626 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 Tg626 mice in a 55-day experiment (Fig. 2).

DISCUSSION

Ura-blast 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-blast 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 microflora, 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-blast methodology are also auxotrophic for biotin and perhaps for other water-soluble vitamins (Odds, 1979).

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**Fig. 2.** Lethality of wild-type (SC5314) and URA3 mutants (CAI-4, CAF2-1) of C. albicans for colonized gnotobiotic Tg626 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., 2003; 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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