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# Increased Virulence and Competitive Advantage of a/α Over a/a or α/α Offspring Conserves the Mating System of *Candida albicans*

**Shawn R. Lockhart, Wei Wu, Joshua B. Radke, Rui Zhao and David R. Soll**[1]

*Department of Biological Sciences, The University of Iowa, Iowa City, Iowa 52242-1324*

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ABSTRACT

The majority of *Candida albicans* strains in nature are **a**/α and must undergo homozygosis to **a**/**a** or α/α to mate. Here we have used a mouse model for systemic infection to test the hypothesis that **a**/α strains predominate in nature because they have a competitive advantage over **a**/**a** and α/α offspring in colonizing hosts. Single-strain injection experiments revealed that **a**/α strains were far more virulent than either their **a**/**a** or α/α offspring. When equal numbers of parent **a**/α and offspring **a**/**a** or α/α cells were co-injected, **a**/α always exhibited a competitive advantage at the time of extreme host morbidity or death. When equal numbers of an engineered **a**/**a**/α2 strain and its isogenic **a**/**a** parent strain were co-injected, the **a**/**a**/α2 strain exhibited a competitive advantage at the time of host morbidity or death, suggesting that the genotype of the mating-type (*MTL*) locus, not associated genes on chromosome 5, provides a competitive advantage. We therefore propose that heterozygosity at the *MTL* locus not only represses white-opaque switching and genes involved in the mating process, but also affects virulence, providing a competitive advantage to the **a**/α genotype that conserves the mating system of *C. albicans* in nature.

*CANDIDA albicans*, which is diploid, contains a single mating-type locus, *MTL* (Hull and Johnson 1999). While 97% of natural strains are *MTL* heterozygous (**a**/α), 3% are *MTL* homozygous (**a**/**a** or α/α) (Lockhart *et al.* 2002). Only *MTL*-homozygous **a**/**a** and α/α strains can mate (Hull *et al.* 2000; Magee and Magee 2000; Miller and Johnson 2002; Lockhart *et al.* 2003a; Soll 2004). To become *MTL* homozygous, an **a**/α strain undergoes spontaneous *MTL* homozygosis either through loss of one homolog of chromosome 5, which harbors the *MTL* locus, followed by duplication of the retained homolog, or, less frequently, through mitotic crossing over (Wu *et al.* 2005). Expressing a mating type in *C. albicans* is, therefore, markedly different from expressing a mating type in *Saccharomyces cerevisiae*. *S. cerevisiae* possesses three different loci containing mating-type genes, two that are silent (*HML*, *HMR*) and one that is expressed (*MAT*). *HML* contains copies of **a** genes and *HMR* copies of α genes. *MAT* contains either **a** or α genes. Mating-type switching in *S. cerevisiae* occurs by recombination at the *MAT* locus with a copy of the silent locus harboring the alternative mating-type genes (Rine *et al.* 1979; Haber 1998). *S. cerevisiae*, therefore, conserves the alternative genetic information of **a** and α in silent cassettes when expressing either mating type. In contrast, *C. albicans* loses alternative *MTL* information when expressing a mating type.

Genetic studies of strain relatedness have revealed that the population structure of *C. albicans* is primarily clonal, with only hints of recombination (Graser *et al.* 1996; Xu *et al.* 1999; Pujol *et al.* 1993, 2004). Hence, mating most likely is a rare event and as such is an unlikely mechanism for returning *MTL*-homozygous strains to *MTL* heterozygosity. Since natural *MTL*-heterozygous strains can and do spontaneously generate *MTL* homozygotes (Lockhart *et al.* 2002; Pujol *et al.* 2003; Wu *et al.* 2005), but *MTL* homozygotes rarely replenish *MTL* heterozygotes, why do *MTL* homozygotes not accumulate and predominate in nature? Why are only 3% of natural strains *MTL* homozygous? One possible explanation is that *MTL* heterozygotes are more competitive than *MTL* homozygotes in natural settings. Here, we have tested this hypothesis, first by comparing virulence between **a**/α strains and their **a**/**a** and α/α offspring in the mouse model for systemic infection, and second by testing for competition by co-injecting mice with mixtures of **a**/α cells and either their **a**/**a** or α/α offspring cells. Our results demonstrate that three unrelated parental **a**/α strains are far more virulent than their **a**/**a** or α/α offspring, as measured by the time of extreme host morbidity or death in the mouse model for systemic infection, and that for four unrelated strains, **a**/α predominates at the time of extreme host morbidity or death in animals co-injected with equal volumes of **a**/α and either their **a**/**a** or α/α

[1]*Corresponding author:* Department of Biological Sciences, Room 302 BBE, University of Iowa, Iowa City, IA 52242.
E-mail: david-soll@uiowa.edu

**TABLE 1**

**Strains used in this study**

| Strain | Reference |
|---|---|
| P37037**a**/α | Pujol *et al.* (2003) |
| P37037α/α-1 | Lockhart *et al.* (2002) |
| P37037α/α-2 | Wu *et al.* (2004) |
| P37037α/α-3 | Wu *et al.* (2004) |
| P37037**a**/**a**-1 | Wu *et al.* (2004) |
| P37039**a**/α | Pujol *et al.* (2003) |
| P37039α/α-1 | Lockhart *et al.* (2002) |
| P75063**a**/α | Pujol *et al.* (2002) |
| P75063**a**/**a**-1 | Lockhart *et al.* (2002) |
| P34048**a**/α | Wu *et al.* (2004) |
| P34048α/α-1 | Wu *et al.* (2004) |
| P34048**a**/**a**-1 | Wu *et al.* (2004) |
| P37005**a**/**a** | Lockhart *et al.* (2002) |
| P37005**a**/**a**/α2 | This study |

offspring. Furthermore, an engineered **a**/**a**/α2 strain had a competitive advantage at the time of extreme host morbidity or death when co-injected with its isogenic **a**/**a** parent strain in the mouse model, suggesting that the *MTL* genotype, not associated genes on chromosome 5, is responsible for the competitive advantage. We propose that the *MTL* genotype regulates *C. albicans* virulence and that the competitive advantage of the **a**/α genotype maintains **a**/α as the predominant genotype in nature, hence contributing to the conservation of the mating system.

## MATERIALS AND METHODS

**Strain maintenance and culture conditions:** The origins of the strains used in this study are presented in Table 1. Since cells can switch from white to opaque only when they are *MTL* homozygous, spontaneous *MTL*-homozygous offspring of each parent **a**/α strain were isolated by screening for opaque colonies on nutrient agar plates as previously described (Lockhart *et al.* 2002). The *MTL* genotypes of **a**/**a** or α/α offspring were immediately verified by PCR with primers for *MTL***a**1 and *MTL*α2, as described below. All strains were maintained as glycerol stocks at −70°. For experimental purposes, cells from glycerol stocks were grown at 25° on agar plates containing the nutrient composition of Lee's medium (Lee *et al.* 1975) modified according to Bedell and Soll (1979). The agar was further supplemented with phloxine B to identify white-phase colonies (Anderson and Soll 1987) in *MTL*-homozygous populations.

**Virulence in a systemic mouse model:** Injections were performed as described by Kvaal *et al.* (1997). Five-day-old white colonies were selected from each strain for analysis. Cells from a colony were grown to late log phase in modified Lee's medium, washed twice in sterile phosphate buffered saline (PBS; 3 mm KCl, 137 mm NaCl, 2 mm $KH_2PO_4$, 7 mm $NaH_2PO_4$, pH 7.4) and resuspended in PBS at a concentration of $4 \times 10^6$ cells/ml. If two strains were mixed 1:1, the concentration of each was $2 \times 10^6$ cells/ml. Cell densities were estimated using a hemocytometer. Cell phenotype was checked microscopically to be sure >99% of cells were in the white phenotype (Anderson and Soll 1987; Slutsky *et al.* 1987). Six- to 8-week-old female ND4 mice (Harlan Sprague, Madison, WI) weighing 21–26 g were injected through their tail vein with $1 \times 10^6$ cells. Mice were examined every day. When a mouse showed the first signs of illness (*i.e.*, tremors, hunched back), which we will refer to as "extreme morbidity," it was euthanized using $CO_2$. A previous study showed that death followed the selected moribund symptoms by 1 day or less (Kvaal *et al.* 1997). In the majority of cases, mice died. Either one or both kidneys were removed and ground in 2 ml of sterile PBS in a sterile mortar and pestle. Aliquots of each kidney macerate were plated on modified Lee's medium containing phloxine B (Anderson and Soll 1987) and incubated at 25° for 5 days. In control experiments in which mixtures of **a**/α and *MTL*-homozygous offspring were injected, we obtained similar results for alternative kidneys of the same animal and alternative organs (kidney *vs.* liver) (see Table 4). Therefore, using either one kidney or two kidneys ground together provided similar results.

**PCR analysis:** Following growth, 50 individual colonies were picked with sterile toothpicks and individually streaked on YPD (2% dextrose, 2% peptone, 1% yeast extract) agar plates. After 2 days, individual round colonies were picked, streaked on fresh nutrient agar plates to assure clonality, and grown for 2 days. Cells were then resuspended in 20 μl of sterile water in a 250-μl microfuge tube. The tube was heated to 94° for 6 min and then placed in a −70° freezer. To initiate a polymerase chain reaction (PCR) assay, the preparation was pelleted and 4 μl of cell supernatant, 2.5 μl of 10 × PCR buffer provided by the manufacturer (Invitrogen, Carlsbad, CA), 0.75 μl 50 mm $MgCl_2$, 0.25 μl 10 mm dNTPs, 0.5 μl each of 5 μm forward and reverse primers, 0.15 μl Taq DNA polymerase (Invitrogen), and water were added to a fresh tube to a final volume of 25 μl. After an initial denaturation step at 94° for 5 min, the following reaction conditions were used for 40 cycles: 94° for 1 min, 47° for 1 min, and 68° for 1 min. A final elongation step at 72° for 7 min completed the reaction. If a preparation gave no PCR result, it was reanalyzed by PCR using fresh colonies.

**Generation of an a/a/α2 strain:** To generate an **a**/**a**/α2 strain from the natural **a**/**a** strain P37005, which did not contain any auxotrophic markers, a plasmid that contained the mycophenolic acid resistance allele was first constructed. The mycophenolic acid resistance allele of the *IMH3* gene was released by *Xho*I digestion from plasmid p3408 (Beckerman *et al.* 2001), a generous gift from P. T. Magee at the University of Minnesota. The sticky ends of the 2.7-kb fragment were blunt ended and then subcloned into the plasmid pCaExp at the *Bgl*II site to generate the plasmid pCaM. pCaExp (Care *et al.* 1999), a generous gift from P. Sudbery at Sheffield University, is an integrative plasmid designed to allow expression of a selected gene under the control of the *MET3* promoter. It contains the *C. albicans RP10* gene for site-directed integration via homologous recombination. *MTL*α2 was cloned by PCR using the primers 5′-ATT GGA TCC ATG AAT TCA CAT CTG GAG GCA-3′ and 5′-ATT CTG CAG TTA ACC TGT TAA TAG CAA AGC-3′, which contain engineered *Bam*HI and *Pst*I sites, respectively. Following digestion with *Bam*HI and *Pst*I, the *MTL*α2 fragment was ligated to *Bam*HI- and *Pst*I-digested pCaM, downstream of the *MET3* promoter. The *MET3* promoter was employed because it is leaky and allowed expression of *MTL*α2, as demonstrated by RT-PCR (see results). Sequence and orientation of the resulting plasmid pCaMα2 were confirmed by sequencing (data not shown).

To generate an **a**/**a**/α2 strain, 25 μg of pCaMα2 were linearized at the *Nco*I site within the *RP10* gene, which encodes a ribosomal protein (Care *et al.* 1999). The *RP10* gene has been identified as a neutral site for integration (Swoboda *et al.*

**TABLE 2**

**Primers used for RT-PCR**

| Gene | Name | Sequence |
|---|---|---|
| *MTL***a**1 | **a**1*Sma*IFT | 5′-ATC CCC CGG GAA TGA ACT CAG AAA TAG A-3′ |
| | **a**1*Sma*IR | 5′-TCC CCC GGG CTA GGT TGA ATT TGA ACT-3′ |
| *MTL*α2 | α2*Bam*HIF | 5′-ATT GGA TCC ATG AAT TCA CAT CTG GAG GCA-3′ |
| | α2*Pst*IR | 5′-ATT CTG CAG TTA ACC TGT TAA TAG CAA AGC-3′ |
| *MTL*α1 | α1*Bam*H1F | 5′-CAG GGA TCC TGG CTT CAA CAG ATA TGG GAA-3′ |
| | α1*Pst*IR | 5′-TAA CTG CAG TTA CTT CAT TAT GTA AAC ATC-3′ |
| *HIS3* | *HIS3*F | 5′-ATG TCA CGA GAA GCT TTA-3′ |
| | *HIS3*R | 5′-TCT ACT CAA TGC TTC ATC-3′ |

1995). The linearized DNA preparation was transformed into strain P37005, a natural **a**/**a** strain (Lockhart *et al.* 2002), using the lithium acetate method (Schiestl and Gietz 1989). Transformants were selected for mycophenolic acid resistance by growing cells on minimal medium supplemented with 5 μg/ml of mycophenolic acid (Sigma, St. Louis). Selected transformants were confirmed to be single-copy integrations at the *RP10* locus by Southern analysis. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to confirm *MTL*α2 gene expression.

**RT-PCR:** To analyze expression of *MTL***a**1, *MTL*α2, *MTL*α1 and the constitutively expressed gene *HIS3*, RT-PCR was performed according to the protocol of the Access RT-PCR System provided by Promega (Madison, WI). The primers are described in Table 2. RNA was extracted according to methods previously described (Lockhart *et al.* 2003a,b). Prior to RT-PCR, RNA samples were treated with RNase-free DNase (RQ1, Promega) at 37° for 1 hr to remove DNA contamination. One-tenth of a microgram of RNA was used as template for each reaction. Reverse transcription was performed at 48° for 45 min, immediately followed by denaturation at 94° for 5 min. The denatured template was then subjected to the following reaction regimen: 40 cycles at 94° for 30 sec, 45° for 1 min, and 68° for 2 min. The final elongation reaction was performed at 68° for 7 min. RT-PCR of the constitutively expressed *C. albicans HIS3* gene served as a control. All PCR and RT-PCR reactions were performed in a Programmable Thermal Block II thermal cycler (Lab-line Instruments, Melrose Park, IL).

## RESULTS

**a/α strains are more virulent than their a/a or α/α offspring:** Kill curves were generated for mice injected with **a**/α strains alone or with their **a**/**a** or α/α offspring alone. The three tested parent strains and their offspring were as follows: P37037**a**/α, P37037**a**/**a**-1, and P37037α/α-1; P37039**a**/α and P37039α/α-1; and P75063**a**/α and P75063**a**/**a**-1. In each of the three tested combinations, the parent **a**/α strain killed mice much faster than the *MTL*-homozygous offspring, whether **a**/**a** or α/α (Figure 1, A–C). While the **a**/α parent strains P37037**a**/α, P37039**a**/α, and P75063**a**/α caused 50% host death after 10, 12, and 5 days, respectively, their *MTL*-homozygous offspring caused only 0%, 10%, and 20% host death, respectively, after 17 days (Figure 1, A–C, respectively). Natural **a**/α strains were, therefore, consistently far more virulent in this model than their *MTL*-homozygous offspring.

**Stability of *MTL* heterozygotes in the mouse model:** To conclude that **a**/α strains were more virulent than **a**/**a** or α/α strains in single-strain infections, and to justify competition experiments between **a**/α and either **a**/**a** or α/α strains, we first had to demonstrate that the **a**/α genotype was stable *in vivo*—*i.e.*, that **a**/α strains did not undergo high levels of homozygosis after injection into a mouse. To assess stability, cells of strains P37037**a**/α, P37039**a**/α, and P75063**a**/α were individually injected into mice. At the time of host death, 50 clones from the kidney macerate of each mouse were analyzed for *MTL* zygosity by PCR. One-hundred percent of the clones derived from kidneys of mice injected individually with strains P37037**a**/α and P37039**a**/α were **a**/α (Table 3). In the case of strain P75063**a**/α, 100% and 86% were **a**/α in the two test mice (Table 3). P75063 was previously observed to undergo very high rates of spontaneous *MTL* homozygosis *in vitro* (Wu *et al.* 2005). These results demonstrate that the **a**/α genotype is relatively stable during the course of a mouse model experiment.

**Co-injection of a/α and their a/a or α/α offspring:** The kill curves for mice injected with single strains (Figure 1) revealed that **a**/α strains were more virulent than their **a**/**a** or α/α offspring. To test for competitiveness, equal numbers of **a**/α and either an **a**/**a** or an α/α offspring were co-injected into mice, and the proportions of *MTL* genotypes were tested at the time of extreme host morbidity or death by PCR analysis of ~50 random clones, which were isolated from the kidneys of test animals. Four unrelated **a**/α strains and their respective offspring were analyzed (Table 4). For the **a**/α strain P37037**a**/α, one **a**/**a** and three α/α offspring were analyzed in mixtures with the **a**/α parent strain, and for strain P34048**a**/α, one **a**/**a** and one α/α offspring were analyzed in mixtures with the **a**/α parent strain. For two additional **a**/α strains, P37039**a**/α and P75063**a**/α, one α/α and one **a**/**a** strain, respectively, were analyzed in mixture. Multiple mice were tested for each mixture. In all eight tested mixtures, the genotype of the majority

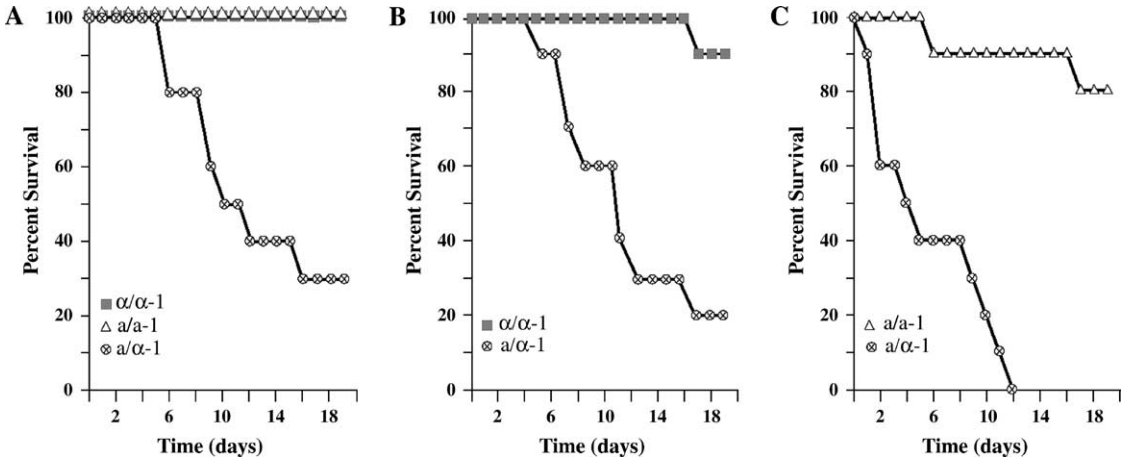


FIGURE 1.—*MTL*-heterozygous (**a**/α) strains of *Candida albicans* are more virulent than their **a**/**a** and α/α offspring in the mouse model for systemic infection. For each strain, 10 mice were each injected with $1 \times 10^6$ cells. The percentage of surviving animals is plotted as a function of time for each tested strain. (A) P37037, (B) P37039, and (C) P75063 are independent **a**/α strains. The **a**/**a** and α/α offspring appeared spontaneously *in vitro*.

of yeast at the time of extreme host morbidity or death was **a**/α (Table 4). As controls in one combination (P37037**a**/α + P37037α/α-1), the two kidneys of one animal (2-1 and 2-2) were macerated separately and a PCR analysis was performed on yeast clones from each. In another combination (P37037**a**/α + P37037 α/α-3), yeast clones from the liver were tested. In all of these controls, **a**/α again predominated (Table 4). These results indicate that when mixtures of **a**/α cells and either their **a**/**a** or α/α offspring are co-injected with equal cell numbers into a host, the **a**/α parent strain had a competitive advantage in every case at the time of host death. Growth experiments in liquid nutrient medium or on agar failed to reveal a growth advantage for **a**/α cells, at least *in vitro* (data not shown).

**Co-injection of a/a and α/α offspring:** Experiments in which **a**/α cells and their *MTL*-homozygous offspring were co-injected into the mouse model revealed that cells of every tested **a**/α strain exhibited a competitive advantage in the infection at the time of host death. These results revealed no differences between **a**/**a** and α/α offspring. To test for such differences, **a**/**a** and α/α cells from the same **a**/α parent were co-injected and colonization of the kidneys analyzed either at the time of extreme host morbidity or death or after 17 days, when surviving animals were killed. Three to six animals were injected in each cross, and 50 clones were analyzed for *MTL* genotype at the time of extreme host morbidity or death. Crosses were performed between P34048**a**/**a**-1 and P34048α/α-1 and between P37037**a**/**a**-1 and P37037 α/α-1, P37037α/α-2, or P37037α/α-3. In the case of P34048, the proportions of **a**/**a** and α/α cells at the time of host death were 38 ± 14% and 62 ± 14%, respectively. For the mixture P37037**a**/**a**-1 and P37037 α/α-1, the proportions of **a**/**a** and α/α cells at the time of host death or killing were 1 ± 2% and 99 ± 2%, respectively. However, for the mixtures P37037**a**/**a**-1 and either P37037α/α-2 or P37037α/α-3, the proportions of **a**/**a** and α/α cells at the time of host death in both cases were 100 and 0%, respectively. While P37037 α/α-1 was spontaneously generated by mitotic recombination, P37037α/α-2 and P37037α/α-3 were spontaneously generated by the loss of one chromosome 5 homolog followed by duplication of the retained homolog (WU *et al.* 2005). Although these results demonstrate that, within a strain, either an **a**/**a** or an α/α derivative can have a competitive advantage in a mixed infection, there was no universal advantage by either *MTL* genotype over the other, as there was for the *MTL***a**/α genotype over *MTL*-homozygous offspring.

**Isogenic a/a *vs.* a/a/α2 competition:** Our results demonstrated that **a**/α strains are more competitive than their **a**/**a** or α/α offspring in mixed systemic infections. Our results, however, did not distinguish between an advantage due to the heterozygosity of genes linked

**TABLE 3**

***MTL* heterozygosity (a/α) is relatively stable in the mouse model for systemic infection**

| Experiment | Strain | No. of clones analyzed[a] | % clones | | |
|---|---|---|---|---|---|
| | | | **a**/α | **a**/**a** | α/α |
| 1 | P37037 | 50 | 100 | 0 | 0 |
| 2 | P37037 | 50 | 100 | 0 | 0 |
| 3 | P37039 | 50 | 100 | 0 | 0 |
| 4 | P37039 | 50 | 100 | 0 | 0 |
| 5 | P75063 | 50 | 100 | 0 | 0 |
| 6 | P75063 | 50 | 86 | 14 | 0 |

[a] Clones were taken from the kidney of the injected mouse at the time of extreme host morbidity or death and analyzed for *MTL* zygosity by PCR.

**TABLE 4**

**a/α strains are more competitive than a/a or α/α offspring in the mouse model for systemic infection**

| Strain combination[a] | Mouse | No. clones analyzed[b] | % genotype[c] | | |
|---|---|---|---|---|---|
| | | | **a**/α | **a**/**a** | α/α |
| P37037**a**/α + P37037**a**/**a**-1 | 1 | 49 | 96 | 4 | 0 |
| | 2 | 48 | 100 | 0 | 0 |
| | 3 | 50 | 98 | 2 | 0 |
| | Mean ± SD | | 98 ± 2 | 2 ± 2 | 0 |
| P37037**a**/α + P37037α/α-1 | 1 | 50 | 64 | 0 | 36 |
| | 2-1[d] | 50 | 82 | 0 | 18 |
| | 2-2[d] | 50 | 84 | 0 | 16 |
| | 3 | 50 | 88 | 0 | 12 |
| | Mean ± SD | | 80 ± 11 | 0 | 20 ± 11 |
| P37037**a**/α + P37037α/α-2 | 1 | 50 | 100 | 0 | 0 |
| | 2 | 50 | 96 | 0 | 4 |
| | Mean ± SD | | 98 | 0 | 2 |
| P37037**a**/α + P37037α/α-3 | 1 | 49 | 100 | 0 | 0 |
| | 2 | 50 | 100 | 0 | 0 |
| | 3 | 37 | 92 | 0 | 8 |
| | 4[e] | 45 | 91 | 0 | 9 |
| | Mean ± SD | | 94 ± 5 | 0 | 6 ± 5 |
| P37039**a**/α + P37039α/α-1 | 1 | 50 | 98 | 0 | 2 |
| | 2 | 50 | 100 | 0 | 0 |
| | 3 | 50 | 100 | 0 | 0 |
| | Mean ± SD | | 99 ± 1 | 0 | 1 ± 1 |
| P34048**a**/α + P34048**a**/**a**-1 | 1 | 47 | 100 | 0 | 0 |
| | 2 | 47 | 98 | 2 | 0 |
| | 3 | 50 | 94 | 6 | 0 |
| | Mean ± SD | | 97 ± 3 | 3 ± 3 | 0 |
| P34048**a**/α + P34048αα-1 | 1 | 47 | 100 | 0 | 0 |
| | 2 | 50 | 100 | 0 | 0 |
| | 3 | 48 | 88 | 0 | 12 |
| | Mean ± SD | | 96 ± 7 | 0 | 4 ± 7 |
| P75063**a**/α + P75063**a**/**a**-1 | 1 | 49 | 98 | 2 | 0 |
| | 2 | 46 | 87 | 13 | 0 |
| | Mean ± SD | | 93 | 8 | 0 |

[a] P37037**a**/**a**-1, P37037α/α-2, P37037α/α-3, P37039α/α-1, P34048**a**/**a**-1, and P34048α/α-1 were spontaneously generated by loss of one chromosome 5 homolog followed by duplication of the retained homolog, while P37037α/α-1 was spontaneously generated by mitotic recombination (Wu *et al.* 2005).

[b] Clones were taken from the kidney of the injected mouse at the time of extreme host morbidity or death and analyzed for *MTL* genotype by PCR.

[c] Mean ± SD was computed for each combination.

[d] Mouse 2-1 and 2-2 represent different kidneys of the same mouse.

[e] Mouse 4 is actually the liver from mouse 3 macerated and analyzed.

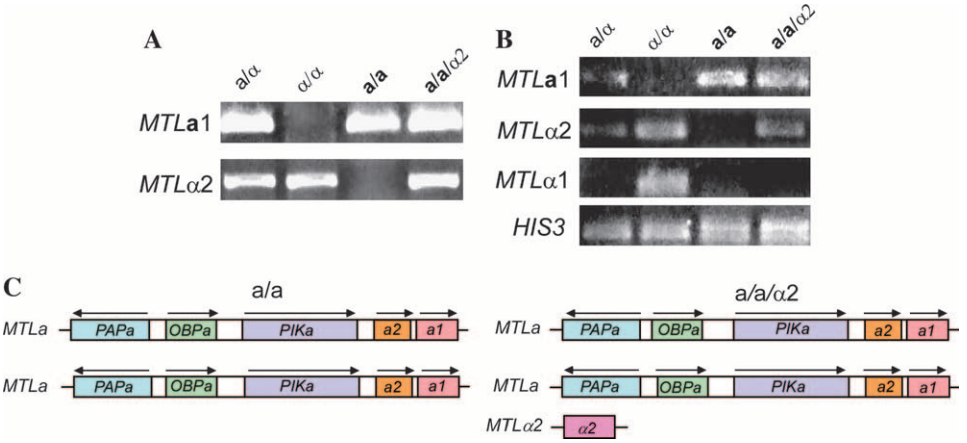


FIGURE 2.—Strain P37005 **a**/**a**/α2 was generated from the natural *C. albicans* strain P37005 **a**/**a**, using a dominant selection marker, the mycophenolic resistance allele of the *IMH3* gene (BECKERMAN *et al.* 2001). (A) *MTL* genotyping by PCR demonstrated the addition of *MTL*α2 in the **a**/**a**/α2 strain. (B) RT-PCR demonstrated that while the **a**/**a** parent expressed only *MTL***a**1, the **a**/**a**/α2 derivative expressed *MTL***a**1 and *MTL*α2. The constitutively expressed gene *HIS3* was assayed as a control. (C) The **a**/**a**/α2 derivative contains two copies of the entire *MTL***a** locus and one copy of the *MTL*α2 gene.

to the *MTL* locus on chromosome 5 (WU *et al.* 2005) *vs.* an advantage due solely to heterozygosity at the *MTL* locus. To distinguish between these alternatives, we created an isogenic **a**/**a**/α2 strain from the **a**/**a** strain P37005 (Figure 2). This strain was generated by integrating a plasmid containing *MTL*α2 under control of the *MET3* promoter and a dominant selection marker, the mycophenolic resistance allele of the *IMH3* gene (BECKERMAN *et al.* 2001) at the neutral *RP10* gene (SWOBODA *et al.* 1995; CARE *et al.* 1999). Hence, neither parent nor offspring harbored auxotrophic genes, a requirement for mouse model experiments. An RT-PCR analysis of the low level *MTL***a**1 and *MTL*α2 transcripts from the parental and transformed strain revealed that both *MTL***a**1 and *MTL*α2 were expressed in the **a**/**a**/α2 derivative (Figure 2B). When P37005**a**/**a** and P37005**a**/**a**/α2 cells were co-injected into the mouse model, **a**/**a**/α2 cells exhibited a competitive advantage at the time of host death (Table 5). These results suggest that it is the *MTL* genotype, not allelism of genes linked to *MTL* on the chromosome 5 homologs, that provides the competitive advantage exhibited by **a**/α strains over their **a**/**a** or α/α offspring.

## DISCUSSION

Our results first demonstrate that **a**/**a** and α/α offspring are far less virulent than their parental **a**/α strains when tested alone for the time of extreme host morbidity or death in the mouse model of systemic infection. This held true for three unrelated **a**/α strains and their *MTL*-homozygous offspring. It also held true for strains from different clades. P37037 is a member of clade I, while P75063 is a member of clade SA (BLIGNAUT *et al.* 2002; PUJOL *et al.* 2002, 2003; SOLL and PUJOL 2003). More importantly, in mixing experiments of four different **a**/α strains with their **a**/**a** or α/α offspring, the **a**/α parent strain in each case exhibited a strong competitive advantage at the time of extreme host morbidity or death. In the case of strain P37037, this held true for an α/α offspring (P37037α/α-1) that was generated spontaneously by mitotic recombination along chromosome 5, and for α/α offspring (P37037α/α-2 and P37037α/α-3) that were generated spontaneously by loss of one chromosome 5 homolog followed by duplication of the retained homolog (WU *et al.* 2005). Mixing experiments of **a**/**a** and α/α offspring of the same parent strains revealed no universal advantage for either

**TABLE 5**

**The a/a/2 derivative of strain P37005a/a-1 has a competitive advantage in the mouse model for systemic infection**

| Strain combination | Mouse | No. clones analyzed[a] | % genotype reserved | | |
|---|---|---|---|---|---|
| | | | α/α | **a**/**a** | **a**/**a**/α2 |
| P37005**a**/**a**-1 + P37005**a**/**a**/α2 | 1 | 50 | 0 | 34 | 66 |
| | 2 | 49 | 0 | 4 | 96 |
| | 3 | 47 | 0 | 6 | 94 |
| | Mean ± SD | | | 15 ± 17 | 85 ± 17 |

[a] Clones were taken from the kidney of the injected mouse at the time of extreme host morbidity or death and analyzed for *MTL* genotype by PCR.

one, as we found for the **a**/α genotype when mixed with *MTL*-homozygous offspring.

Two alternative explanations could explain the increased virulence and competitive advantage of **a**/α strains over their **a**/**a** or α/α offspring in the mouse model. First, heterozygosity at the *MTL* locus alone may be the basis. *MTL* heterozygosity has been demonstrated to regulate genes associated with the mating process and to suppress phenotypic switching (Lockhart *et al.* 2002, 2003; Miller and Johnson 2002). Our combined results suggest that *MTL* heterozygosity is also involved in the regulation of genes that confer virulence and a competitive advantage in colonization. Alternatively, virulence and competitiveness could be conferred by other genes linked to the *MTL* locus along chromosome 5. A recent analysis revealed heterozygosity for a number of genes other than the *MTL* locus along chromosome 5, and association of particular alleles of these genes with either *MTL***a** or *MTL*α (Wu *et al.* 2005). In this scenario, *MTL***a**-linked and *MTL*α-linked alleles of different genes would combine to confer the **a**/α advantage. To test between these alternatives, we engineered an isogenic **a**/**a**/α2 strain from an **a**/**a** strain, co-injected the transformant and parent strain into mice, and analyzed the genetic composition of infecting yeast at the time of extreme host morbidity or death. The **a**/**a**/α2 strain exhibited a competitive advantage, supporting the hypothesis that the **a**/α genotype, not *MTL***a**-linked or *MTL*α-linked genes, confers virulence and the competitive advantage of **a**/α strains over *MTL*-homozygous offspring in host colonization. Further experiments are now in progress to test this hypothesis.

Although we found that in mixed infections, **a**/α strains have a strong competitive advantage over either their **a**/**a** or α/α offspring, and that **a**/α strains are far more virulent than their *MTL*-homozygous offspring in the mouse model, we also know that ~3% of natural strains are *MTL* homozygous and therefore are successful in nature. Some of these strains, such as strain WO-1, which is α/α (Lockhart *et al.* 2002), are quite virulent in the mouse model for systemic infection (Kvaal *et al.* 1997), suggesting that they have overcome the loss of *MTL* heterozygosity, presumably by compensatory changes in genes other than those at the *MTL* locus. However, the great majority of strains in nature are **a**/α (Lockhart *et al.* 2002), suggesting a general advantage to this genotype. We therefore propose that *MTL* heterozygosity in *C. albicans* plays a fundamental role not only in suppressing α-specific and "haploid"-specific genes (Johnson 2003) and phenotypic switching (Lockhart *et al.* 2002; Miller and Johnson 2002), but also in virulence. We further suggest that *MTL* heterozygosity, presumably through the regulation of genes involved in host colonization, provides a competitive advantage to **a**/α cells over *MTL*-homozygous offspring, which conserves **a**/α cells and hence the mating system in nature.

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