

Reevaluation of the Role of *HWP1* in Systemic Candidiasis by Use of *Candida albicans* Strains with Selectable Marker *URA3* Targeted to the *ENO1* Locus

Paula Sundstrom,^{1,2*} Jim E. Cutler,^{3†} and Janet F. Staab¹

Department of Molecular Virology, Immunology, and Medical Genetics, The Ohio State University College of Medicine and Public Health,¹ and Department of Microbiology, The Ohio State University,² Columbus, Ohio 43210, and Department of Microbiology, Montana State University, Bozeman, Montana 59717³

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Previous evaluation of *HWP1* in systemic candidiasis in CBA/J mice was done with *Candida albicans* strains with differing genetic locations of *URA3* as a result of Ura-blaster mutagenesis. In this study, the presence of *HWP1* and the location of *URA3* contributed to the severity of murine systemic candidiasis in BALB/c mice.

In previous work (8), the *HWP1* gene was required for virulence in systemic candidiasis. A perplexing observation from this study was the suggestion of a trend of increased survival, or slight loss of virulence, of mice given CAH7 (*HWP1/hwp1*) compared to that of mice given CAHR3 (*HWP1/hwp1* revertant). The survival differences were apparently not related to differences in *HWP1* gene expression as the amounts of *HWP1* mRNA and protein were equivalent for the two strains. Moreover, the strains did not differ in growth rates. A difference between strains CAH7 and CAHR3 is the location of the selectable marker gene *URA3*. The *URA3* gene is within the open reading frame of *HWP1* in CAH7 but within *ENO1* in CAHR3.

The use of the Ura-blaster technique in *Candida albicans* (2) results in both inactivation of a gene of interest and ectopic placement of *URA3* within the gene. The observation that *C. albicans* auxotrophic mutants that cannot produce orotidine 5'-monophosphate (OMP) decarboxylase because they lack the *URA3* gene are not pathogenic (4) suggests that if reduced levels of OMP decarboxylase were to be produced in vivo, intermediate virulence might result. Placement of the *C. albicans* *URA3* gene at a locus other than the *URA3* locus during implementation of the Ura-blaster technique has been shown to result in reduced OMP decarboxylase activities in vitro, and the levels of reduction were different for different loci. However, a definitive relationship between OMP decarboxylase enzyme activity in in vitro cultures and virulence was not revealed (6). These studies raised the possibility that the genetic location of the *URA3* gene affects differential in vivo growth rates of strains CAH7 and CAHR3, leading to a trend of decreased virulence of CAH7.

The goals of the present study were twofold. The first was to

make the expression of the *URA3* gene independent of positional effects arising from placement at the *HWP1* locus. The second goal was to be able to compare *C. albicans* strains that are identical with regard to the location of the *URA3* gene. Two new *C. albicans* strains with disruptions at the *HWP1* locus were created by introducing a DNA fragment with *eno1::URA3* (7) into the Ura⁻ strains CAH7-1 (*HWP1/hwp1*) and CAH7-1A1 (*hwp1/hwp1*) (8). Previous studies with a strain bearing a *URA3* disruption of one of four *ENO1* homologues did not reveal reduced growth on pyruvate or glucose (7) or a noticeable reduction in *ENO1* mRNA levels (7, 8), and germ tube formation was unaffected (1, 7).

Ura⁺ transformants were initially screened by PCR for homologous recombination of the *eno1::URA3* fragment at *ENO1*, and positive transformants were confirmed by Southern blot analysis (data not shown). These strains, CAH7-1E1 (*HWP1/hwp1*) and CAH7-1A1E2 (*hwp1/hwp1*), are comparable to the strains, CAH7 and CAH7-1A, used in the previous experiment. However, *URA3* was targeted to the *ENO1* locus in the newly constructed strains as in the revertant strain CAHR3 used in the previous study (Table 1). No differences in growth rates between strains were found in yeast nitrogen base medium without uridine. In addition to the new strains, CAH49, an additional *HWP1/hwp1* heterozygote with *URA3* integrated into the *HWP1* locus, was included along with CAH7.

The effect of *HWP1* in systemic candidiasis was reevaluated by comparing the survival curves of mice given isogenic strains differing in *HWP1* copy number but identical in the location of the *URA3* gene. Survival curves of mice given the homozygous *hwp1/hwp1* mutant CAH7-1A1E2 were compared to those of mice given the heterozygous *HWP1/hwp1* and revertant strains CAH7-1E1 and CAHR3, respectively. Each BALB/c mouse (female, 8 weeks old, $n = 5$) was given 0.1 ml of yeast cell suspension (5×10^6 cells/ml) (as per *C. albicans* strain CA-1 in reference 3) in phosphate-buffered saline and observed daily over a 30-day period. The animals used in this study were housed at the Association for Assessment and Accreditation of Laboratory Animal Care-certified Resources Center at Mon-

* Corresponding author. Mailing address: Department of Molecular Virology, Immunology, and Medical Genetics, 333 W. 10th Ave., The Ohio State University College of Medicine and Public Health, Columbus, OH 43210-1239. Phone: (614) 292-5525. Fax: (614) 292-9805. E-mail: sundstrom.1@osu.edu.

† Present address: Research Institute for Children, Children's Hospital, New Orleans, LA 70118.

TABLE 1. Strains used in this study

Strain	Genotype or description	Source or reference
SC5314	Wild type, parent of CAI4	5
CAH7 ^a	<i>HWP1/hwp1::hisG-URA3-hisG</i>	8
CAH49	<i>HWP1/hwp1::hisG-URA3-hisG</i>	8
CAH7-1	<i>HWP1/hwp1::hisG</i>	8
CAH7-1A	<i>hwp1::hisG-URA3-hisG/hwp1::hisG</i>	8
CAH7-1A1	<i>hwp1::hisG/hwp1::hisG</i>	8
CAHR3	<i>hwp1::hisG/HWP1 eno1::URA3</i> (revertant)	8
CAH7-1E1	<i>HWP1/hwp1::hisG eno1::URA3</i>	This study
CAH7-1A1E2	<i>hwp1::hisG/hwp1::hisG eno1::URA3</i>	This study

^a All CAH strains were derived from the Ura⁻ strain CAI4 (2).

tana State University under the National Institutes of Health guidelines for the care and use of laboratory animals. The survival curves showed that virulence of the *hwp1/hwp1* mutant, CAH7-1A1E2, was reduced compared to that of the other strains (Fig. 1A). The differences in survival between CAH7-1A1E2 (*hwp1/hwp1*) and CAH7-1E1 or CAHR3 (both *HWP1/hwp1*) were statistically significant ($P = 0.0018$ and $P = 0.0016$, respectively, as calculated by the log rank test as before [3]). This is in agreement with the result of the previous study and supports the conclusion that *HWP1* is important for systemic candidiasis.

In contrast to results with strain CAH7, the survival curve of the new *HWP1/hwp1* heterozygote, CAH7-1E1, was equivalent to that of the revertant strain CAHR3 (Fig. 1A) and both of these strains were as virulent as wild-type strain SC5314. A trend of reduced virulence relative to CAHR3 was not found. The results strongly suggested that the location of the *URA3* gene at the *HWP1* locus in strain CAH7 contributed to its reduced virulence relative to CAHR3 in the previous study. A role for the genetic location of *URA3* was also shown by differences in survival curves between the *HWP1/hwp1* heterozygote strains that differed in the location of the *URA3* gene. Strains CAH49 and CAH7 (*HWP1/hwp1*), with *URA3* interrupting *HWP1*, were each reduced in virulence compared to CAH7-1E1 (*HWP1/hwp1*), with *URA3* disrupting *ENO1* ($P = 0.0016$ for both strains versus CAH7-1E1) (Fig. 1B). In contrast to the *HWP1/hwp1* heterozygotes, targeting the *URA3* gene to the enolase locus did not increase the virulence of the homozygous *hwp1/hwp1* mutant. Survival curves of mice injected with strains CAH7-1A1E2 and CAH7-1A (both *hwp1/hwp1*) were not different ($P = 0.3915$) (Fig. 1C). The absence of *HWP1* led to reduced virulence that was not enhanced by placing the *URA3* gene at *ENO1*. This result strengthens the conclusion that *HWP1* is important for virulence.

By comparing the virulence of strains with the selectable marker *URA3* positioned identically, we were able to confirm that *HWP1* is important for systemic candidiasis in mice. However, we also showed that the location of the *URA3* selectable marker may influence the in vivo performance of *C. albicans* strains. Although measurement of *URA3* gene activity in vivo is difficult and impractical, the results presented in this study suggest that placing *URA3* at the *HWP1* locus may lead to decreased *URA3* expression in vivo, thereby causing a reduction in virulence compared to strains with *URA3* at the *ENO1* locus. The mechanism leading to the differences in expression based on genetic location are unknown; however, epigenetic

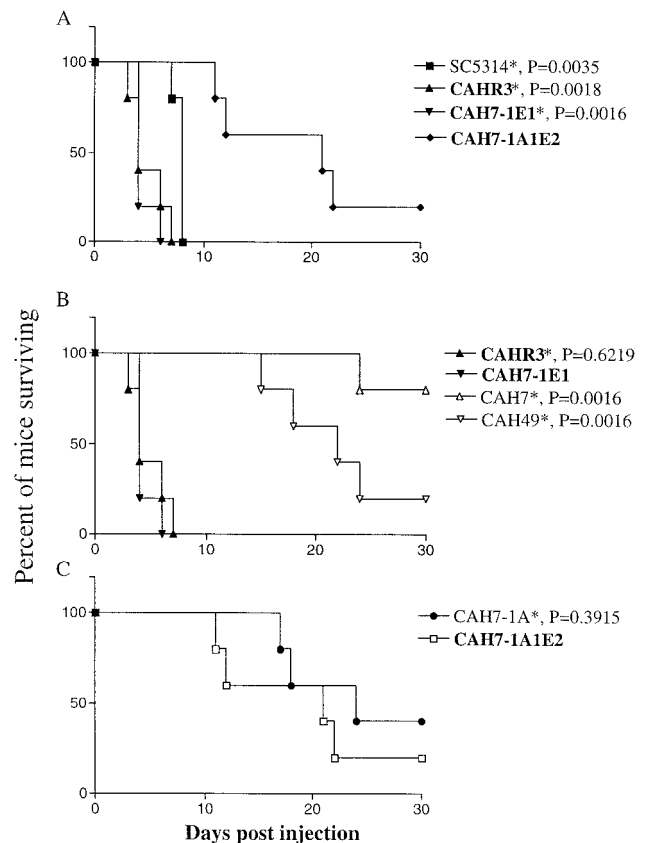


FIG. 1. Survival of BALB/c mice injected intravenously with *C. albicans* strains. (A) Survival of mice injected with *HWP1* mutant strains with *URA3* at the *ENO1* locus (in bold) and with wild-type strain SC5314. The significance of survival differences relative to the *hwp1/hwp1* strain CAH7-1A1E2 by the log rank test is indicated by an asterisk. (B) Survival of mice injected with heterozygous *HWP1/hwp1* strains with *URA3* at the *HWP1* or *ENO1* locus (in bold). The significance of survival differences relative to CAH7-1E1 is indicated by an asterisk. (C) Survival of mice injected with homozygous *hwp1/hwp1* strains with *URA3* at the *HWP1* or *ENO1* locus (in bold). The significance of survival relative to CAH7-1A1E2 is indicated by an asterisk.

regulation of *URA3* at the *HWP1* locus may lead to diminished *URA3* expression. In addition, the induction of *HWP1* during hyphae production may further limit *URA3* levels in vivo. Perhaps these features compromise the cells for Ura3p.

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