Flanking direct repeats of hisG alter URA3 marker expression at the HWP1 locus of Candida albicans

Laura L. Sharkey, Wei-li Liao, Anup K. Ghosh and William A. Fonzi

INTRODUCTION

HWP1 encodes an adhesin of Candida albicans and has been implicated in filamentation and virulence. URA3, an often-used transformation selection marker, is apparently incorrectly expressed when integrated at the HWP1 locus, which results in an attenuated virulence phenotype. Expression of URA3 is compromised by ectopic integration at other loci as well. In contrast, prior studies from the authors’ laboratory had demonstrated that the filamentation deficiency and attenuated virulence of hwp1Δ mutants were fully restored in rescued strains in which URA3 was integrated at the HWP1 locus. This discrepancy prompted a reinvestigation of these mutants. A series of congenic strains were constructed which demonstrated that the filamentation and virulence defects of a homozygous hwp1Δ mutant could be rescued without introduction of a functional HWP1 allele. Despite the absence of detectable differences in URA3 expression, analysis of suppressor mutations suggested that reduced URA3 expression gave rise to the mutant phenotypes. Several independent spontaneous suppressor mutations that restored filamentation to strains of genotype hwp1Δ::hisG-URA3-hisG/hwp1Δ::hisG had acquired a tandem duplication of the hisG-URA3-hisG marker cassette. The hwp1 null mutant and rescued strains differed by the presence or absence of flanking hisG sequence. Substitution of the hisG-URA3-hisG insert of the hwp1 null mutant with URA3 alone largely rescued the filamentation and virulence phenotypes. The presence of a single copy of hisG adjacent to URA3 had no effect. It is concluded that flanking direct repeats of hisG, present as part of a recyclable disruption cassette, negatively influenced URA3 expression and are responsible for the previously reported phenotypes of the hwp1 mutants.

Abbreviation: OMPase, orotidine-5’-monophosphate decarboxylase.

Microbiology (2005), 151, 1061–1071

DOI 10.1099/mic.0.27487-0

Received 15 July 2004
Revised 3 December 2004
Accepted 20 December 2004
Curiously, our prior work had demonstrated that the filamentation deficiency and attenuated virulence of hwp1Δ mutants were fully restored in rescued strains in which URA3 was integrated at the HWP1 locus (Sharkey et al., 1999; Tsuchimori et al., 2000). This seemed at odds with the aforementioned studies and prompted a reinvestigation of these mutants. We demonstrate here that rescue of the phenotypic deficiencies was independent of HWP1. Despite the absence of detectable differences in OMPase expression, analysis of suppressor mutations indicated that reduced URA3 expression gave rise to the mutant phenotypes. However, we show that context rather than the locus of integration was the relevant variable. Specifically, flanking direct repeats of hisG sequences, present as part of a recyclable disruption cassette (Fonzi & Irwin, 1993), negatively influenced disruption cassette (Fonzi & Irwin, 1993), negatively influenced URA3 expression. Preliminary accounts of this work were reported previously [Sharkey, L. L. & Fonzi, W. A. (2001). Presented at the EuroConference on Fungal Virulence Factors and Disease, Seefeld, Austria, 8–13 September 2001. Sharkey, L. L., Liao, W. & Fonzi, W. A. (2002). Abstr. ASM Conferences, Candida and Candidiasis (6th) 2002, S-11, p. 17].

METHODS

Growth conditions. Strains were routinely cultured on YPD or YNB medium (Sherman et al., 1986) at 30 °C. Medium 199 (Gibco-BRL) containing Earle’s salts and glutamine but lacking sodium bicarbonate was buffered with 150 mM Tris, pH 7, at 25 °C. Media were solidified with 1.5% agar and supplemented with 25 μg uridine ml⁻¹ where indicated. Germ tube formation and filamentation on agar-solidified medium 199 were assayed as described previously (Sharkey et al., 1999). The extent of filamentation was quantified by measuring the width of the fringe of hyphae surrounding the colonies following 72 h of incubation. Digital images were analysed using the public domain NIH Image program (developed at the US National Institutes of Health and available at http://rsb.info.nih.gov/nih-image) and all values are the result of at least four independent determinations.

Strain constructions. The strains used in this study are listed in Table 1. Strain LPR-1 was generated by transformation of strain CAL4 with plasmid pELS-12. Plasmid pELS-12 was constructed by cloning a 1.5 kb PstI–EcoRI fragment encompassing nucleotides −2059 to −485 of HWP1 into the like sites of plasmid pELS-5 (Sharkey et al., 1999), thus generating a wild-type HWP1 clone containing a contiguous upstream region from −2059 to −1. The additional upstream sequence was acquired by plasmid rescue from a SacI digest of strain CAL6 (Sharkey et al., 1999) genomic DNA.

Table 1. Strains of C. albicans

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<th>Strain</th>
<th>Parent</th>
<th>Genotype*</th>
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<tr>
<td>CAI4</td>
<td>CAI4</td>
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<td>Sharkey et al. (1999)</td>
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<tr>
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<tr>
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</tr>
<tr>
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<tr>
<td>DDN3-4B1R</td>
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</tr>
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<td>hwp1Δ::hwp1Δ::hisG</td>
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<tr>
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<td>This work</td>
</tr>
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</table>

*All strains are iro1-ura3Δ::imm434/iro1-ura3Δ::imm434 unless otherwise indicated (Fonzi & Irwin, 1993; Garcia et al., 2001).
†One of three independent null mutants previously constructed (Sharkey et al., 1999), but not named in that work.
‡One of three independent heterozygous mutants previously constructed (Sharkey et al., 1999), but not named in that work.
1.4 kb Xbal–Pst fragment of URA3 (Saporito-Irwin et al., 1995) was cloned into the like sites of the vector to yield the final plasmid. Integration of pELS-12 adjacent to the hwp1::hisG allele of CAL4 was targeted by digestion at a unique HindIII site at position +1277 of HWP1 prior to transformation.

Strain CAL8 was constructed by integration of plasmid pELS-15 at the HWP1 locus of strain CAL4 (Sharkey et al., 1999). Plasmid pELS-15 was derived from plasmid pELS-8 (Sharkey et al., 1999) by digestion with EcoRI and ClaI, which removed nucleotides –485 to –4 of HWP1. The ClaI site at nucleotide –4 was created by site-directed mutagenesis of plasmid pELS-5 (Sharkey et al., 1999). Following removal of the –485 to –4 region, a 1.4 kb Xbal–Pst fragment of URA3 (Saporito-Irwin et al., 1995) was cloned into the like sites of the vector to yield the final plasmid. Integration of pELS-15 was targeted by HindIII digestion.

Strain CAL9 was constructed by integration of plasmid pELS-14 at the HWP1 locus of strain CAL4 (Sharkey et al., 1999). Plasmid pELS-14 was derived from plasmid pELS-8 (Sharkey et al., 1999) by digestion with ClaI and BamHI, which removed nucleotides –4 through +552 of HWP1, treatment with Klenow polymerase and ligation. Insertion of the 1.4 kb Xbal–Pst fragment of URA3 resulted in pELS-14. The plasmid was integrated following HindIII digestion.

Strain CAL10 was constructed by integration of plasmid pELS-16 at the HWP1 locus of strain CAL4 (Sharkey et al., 1999). Plasmid pELS-16 contained a 4 bp insertion at position 1365–1346. This places 60 nucleotides are identical to primer HWP1URA3-5 complementary to the coding strand of the 24 nucleotides match the first 24 nucleotides of the HWP1 region of strain CAL4 (Sharkey et al., 1999). Primer hisG5 is complementary to nucleotides 970–950 of URA3. The reactions were monitored at 30 s intervals monitoring the conversion of orotidine 5'-monophosphate decarboxylase (OMPase) activity was measured spectrophotometrically by monitoring the conversion of orotidine 5'-monophosphate to uridine 5'-monophosphate at 285 nm at room temperature (Brody & Westheimer, 1979). The reactions were monitored at 30 s intervals for 30 min using a Beckman DU-68 spectrophotometer. Reaction rates were determined from the initial linear region of the curve using a Δ value of 2.25 × 10^(-6) M^(-1). (Brody & Westheimer, 1979). Linearity with respect to extract was verified for each sample. One unit of enzyme activity was defined as the quantity of enzyme that catalysed conversion of 1 μmol substrate to product min^(-1). Specific activity was defined as units of enzyme activity per mg of extract protein. Protein concentrations were determined using the Bio-Rad Protein Assay with bovine serum albumin as standard. Cell-free protein extracts were prepared from exponential-phase cells. Typically, a 24 h culture was used to inoculate 100 ml of the desired medium to a density of 5 × 10^6 cells ml^(-1). Cultures were incubated for 4–6 h on a rotary shaker then harvested by centrifugation at 4000 g for 5 min at 4 °C. The cell pellet was washed with 5 ml ice-cold assay buffer consisting of 50 mM MES, pH 6.0, 1 mM dithiothreitol and 20% (w/v) glycerol. Washed cells were suspended in 0.5 ml cold assay buffer and broken by two or three passages
through a French press at 8000 p.s.i. (55 MPa). The cell extract was cleared of debris by centrifugation at 8000 g for 15 min at 4 °C. A minimum of three independent extracts was prepared for each strain and culture condition tested.

**Virulence studies.** Virulence was assessed as described previously (Tsuchimori et al., 2000). Strains were cultured 48 h in YNB at 30 °C, harvested by centrifugation, washed with PBS and suspended in a volume of PBS equivalent to the initial culture volume. Following microscopic enumeration, the cell density was adjusted to $5 \times 10^6$ cells ml$^{-1}$. Serial dilutions of the suspension were plated on YPD to determine c.f.u. Female BALB/c mice 9–11 weeks old were inoculated via the tail vein with 200 μl cell suspension containing $1 \times 10^7$ cells. Mice were given food and water ad libitum. Survival was monitored twice daily and moribund mice were killed by asphyxiation with carbon dioxide, as recommended by the American Veterinary Medical Association (AVMA, 2001). Kidneys were aseptically removed and homogenized in PBS. Serial dilutions were plated on YPD and the resulting colonies were analysed by PCR analysis to confirm their genotype. The morphology of candidal cells in the kidney was assessed microscopically following treatment of a portion of the kidney homogenate with 10% KOH (Odds et al., 2000). In each trial ten mice were inoculated with each test strain. Two or three independent trials were conducted for each test strain and CAI12 was included as a positive control in each trial. Statistically identical results ($P > 0.05$) were obtained in each independent trial except as noted in the text. Within-group differences were assessed using Kruskal–Wallis analysis of variance (Campbell, 1989). Paired differences were assessed by both Gehan’s–Wilcoxon and log-ranks tests (Dawson & Trapp, 2001). Calculations were performed using NCSS software (www.ncss.com).

**RESULTS**

**Filamentation is independent of HWP1 expression**

In our prior study, three independent hwp1 deletion mutants exhibited a conditional deficiency in filamentation (Sharkey et al., 1999). When the mutants were cultured on agar plates, development of invasive lateral hyphae was grossly reduced compared to the parental control. However, formation of hyphae in broth culture was normal. The defect was rescued by the introduction of a single copy of HWP1, apparently confirming that the mutant phenotype was related to the loss of HWP1. The URA3 marker was integrated at the HWP1 locus in both sets of strains.

Despite the rescued phenotype, expression of HWP1 was not detected in the rescued strains. As previously reported (Sharkey et al., 1999), HWP1 was abundantly expressed in germ tubes of the control strain, CAI12, and absent in the hwp1 mutant, CAL3 ($hwp1Δ::hisG/hwp1Δ::hisG$) (Fig. 1). However, no HWP1 mRNA was detected in the rescued strain, CAL5 ($hwp1Δ::hisG-URA3-HWP1/hwp1Δ::hisG$) (Fig. 1). The HWP1 clone used in constructing CAL5 contained 485 bp of upstream sequence and apparently lacked the transcriptional control elements needed for expression. When an analogous strain, LPR-1, was constructed with an additional 1550 bp of upstream sequence, HWP1 expression was restored (Fig. 1) and the filamentation defect was similarly rescued (data not shown).

The apparent discord between the rescued phenotype of strain CAL5 and the absence of detectable HWP1 expression had several possible explanations. A spontaneous second-site suppressor mutation that restored filamentation might have been acquired in the course of constructing CAL5. A suppressor was demonstrably absent from strain CAL4 ($hwp1Δ::hisG/hwp1Δ::hisG$), the Ur$^–$ progenitor of CAL5. This was shown by reintroduction of the hisG-URA3-hisG cassette at the HWP1 locus of strain CAL4, ostensibly recreating the original $hwp1Δ$ mutant, strain CAL3 ($hwp1Δ::hisG/hwp1Δ::hisG-URA3-hisG$). The resulting strain was deficient in filamentation, indicating the lack of a suppressor in CAL4. A suppressor may have been acquired at a step subsequent to the isolation of CAL4, but this seemed difficult to reconcile with the fact that three independently constructed ‘rescued’ strains (Table 1, strains CAL5, DDN2-3A1R and DDN3-4B1R) recovered the ability to filament (Sharkey et al., 1999).

Another possibility was that HWP1 expression, though undetected in the Northern hybridization, was nonetheless adequate to restore filamentation. Alternatively, HWP1 may not have been expressed when CAL5 was cultured in broth, the method used for Northern blot samples, but was expressed when cells were cultured on agar. In this regard it should be noted that the rescued phenotype is a filamentation deficiency on agar: the $Δhwp1$ mutant filaments normally in broth. Since expression would be difficult to detect in both situations, the presumptive need for HWP1 expression in rescuing the filamentation defect was assessed by genetic tests. Three strains were constructed in which the structure of the HWP1 locus was identical to that of strain CAL5 except for specific mutations in the reintroduced copy of HWP1 (Fig. 2). In strain CAL8 ($hwp1Δ::hisG-URA3-hwp1Δ(−485−4)/hwp1Δ::hisG$), the entire upstream region of HWP1 between nucleotides −485 and −4 was deleted, but the coding region remained intact. The filamentation defect was rescued in CAL8, demonstrating that the presumptive promoter region of the introduced HWP1 allele was not required. This did...
not rule out the possibility of transcription from an ersatz promoter in the adjacent plasmid sequences. Therefore, strains CAL9 and CAL10 were constructed, in which mutations were introduced into the coding region of \( HWP1 \).

In strain CAL9 (\( hwp1D::hisG-URA3-hwp1Δ(−4,+552)/hwp1Δ::hisG \)) nucleotides −4 to +552 of \( HWP1 \) were removed, eliminating the initiating ATG codon and amino terminus. In strain CAL10 (\( hwp1Δ::hisG-URA3-hwp1::115+4/hwp1Δ::hisG \)) the \( HWP1 \) allele contained a 4 bp insertion starting at nucleotide +115 of the coding region. This frame-shift results in premature translation termination, producing a protein lacking the C-terminal 594 amino acids. Filamentation was restored in both CAL9 and CAL10, indicating that neither the amino terminus nor the carboxy terminus of \( Hwp1p \) was required to rescue the phenotype. As a final test, strain DD27-U1 (\( hwp1Δ::hisG-URA3-hwp1Δ::hisG \)) was constructed, in which the \( URA3 \) locus of the \( hwp1 \) deletion mutants was restored, but no additional \( HWP1 \) sequences were introduced. Filamentation was similarly restored in DD27-U1 (Fig. 2), verifying that a functional allele of \( HWP1 \) was not required to rescue the filamentation defect and suggesting that the \( HWP1 \) allele introduced into the rescued strain CAL5 was probably not expressed. Filamentation of these strains was quantitatively similar. Therefore, the filamentation defect was independent of the \( hwp1 \) deletion. Furthermore, the phenotype did not correlate with the location of the \( URA3 \) marker (Fig. 2), which was present at the \( HWP1 \) locus in both the \( hwp1 \) null mutant and rescued strains.

The filamentation defect maps to \( URA3 \)

Despite the lack of correlation between the locus of integration of \( URA3 \) and the filamentation defect, uridine supplementation restored filamentation to strains CAL3, DDN2-3 and DDN3-4, each of genotype \( hwp1Δ::hisG/hwp1Δ::hisG-URA3-hisG \) (Fig. 2, Fig. 3 and data not shown). Supplementation had no effect on any of the ‘rescued’ strains (Fig. 2). This would seem to imply that a defect in \( URA3 \) expression was responsible for the filamentation defect, similar to the observations of Cheng et al. (2003). However, we found no measurable difference between any of the strains in the specific activity of OMPase, the product of \( URA3 \) (data not shown). The relevance of

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**Fig. 2.** Rescue of the filamentation defect in \( HWP1 \) mutants. The \( HWP1 \) locus of the indicated strains was altered by targeted recombination to produce the genetic arrangements depicted. Boxes represent the coding region of the genes as labelled. Thin horizontal lines indicate chromosomal DNA and the thick horizontal line represents pBSK sequences. Inclined lines mark deleted regions; arrows indicate insertions. Adjacent micrographs display the colony periphery of the corresponding strain after 48 h incubation at 37 °C on 199 medium, pH 7, with or without uridine. The right-hand columns indicate the width of the hyphal fringe of the colonies relative to the control strain CAI12 in the presence or absence of uridine; values are the mean ± SD (\( n \geq 4 \)). The ‘−’ adjacent to CAL3 indicates that no meaningful measure could be made, since this strain produced either no hyphae or a few scattered tufts of short hyphae in the absence of uridine.
the OMPase measurements, which were technically limited to broth-cultured cells rather than cells cultured on agar, wherein the phenotype is expressed, was uncertain. However, we could also not exclude the possibility that uridine supplementation was correcting unsuspected metabolic deficiencies upstream or downstream of uridine biosynthesis, for instance, reducing the draw on defective glutamine pools. In light of these uncertainties, we examined suppressor mutants to gain insight into the genetic defect responsible.

Incubation of CAL3, DDN2-3 or DDN3-4 for 5 to 7 days at 37°C resulted in the spontaneous emergence of hyphal sectors from some colonies (Fig. 3a). Subcultures from these sectors produced filamentous colonies quantitatively indistinguishable from wild-type, while subcultures from the opposing non-filamentous sector retained the characteristic filamentation defect (Fig. 3a).

Cells cultured from four independent filamentous sectors were examined by Southern blot hybridization for alterations in structure of the HWP1 locus. Each had acquired an approximately 2-7 kb insertion, detected in a BstBI plus PshAI digest of genomic DNA hybridized with HWP1 DNA (Fig. 3b). In each case the insertion occurred within the hisG-URA3-hisG-containing allele, not the allele containing hisG (Fig. 3b), indicated by loss of the 6-3 kb hybridization band and acquisition of a 9 kb band. Moreover, recombination between the hisG repeats, as selected on medium containing 5-fluoroorotic acid (Fonzi...
& Irwin, 1993), resulted in loss of the 2·7 kb insert (Fig. 3b), suggesting that the insertion lies between the hisG repeats.

The suppressed and parental mutants were indistinguishable in Southern blots of EcoRI-digested DNA hybridized with HWP1 (not shown). However, hybridization with URA3 detected a 2·7 kb fragment present in the suppressed, but not the parental mutants (Fig. 3c). The size of this fragment, its hybridization with URA3 and the EcoRI restriction pattern were consistent with a tandem duplication of hisG-URA3 as illustrated in Fig. 3(d). PCR analysis verified the presence of the duplication. Non-convergent primers complementary to the ends of URA3 were used to amplify genomic DNA from the parental mutants and suppressed mutants. Because the primers do not converge, no product is expected from an isolated copy of URA3. However, tandem copies were predicted to yield a 1·6 kb PCR product. No product was detected in samples from the parental mutants, but a 1·6 kb product was amplified from the suppressed mutants (Fig. 3d). Additional PCR mapping demonstrated the presence of the intervening hisG sequences in the amplification product (data not shown).

Thus we concluded that the filamentous sectors arose from cells that had acquired a tandem duplication of URA3. In conjunction with the observation that uridine supplementation rescued the phenotype (Fig. 3e), it appeared that URA3 expression is compromised in the original null mutants, but not in the rescued strains.

**URA3 expression is altered by the presence of direct repeats**

A trivial explanation of the foregoing results was that a spontaneous mutation had occurred in the URA3 sequences of the hisG-URA3-hisG clone used to disrupt the HWP1 locus. This possibility was ruled out by introduction into the HWP1 locus of a hisG-URA3-hisG cassette from an independent plasmid, pECEMB-2 (Fonzi & Irwin, 1993). This failed to restore filamentation in any of the three hwp1Δ mutants tested, but supported normal filamentation when integrated at the ECE1 locus (Fonzi & Irwin, 1993, and data not shown).

A primary distinction between those mutants that filamented and those that did not was the presence or absence of direct repeats of hisG flanking URA3. The consequence of these repeats was assessed by insertion of the URA3 marker alone at the identical position within the HWP1 coding region. This was achieved by PCR amplification of URA3 using primers with 5′ complementarity to HWP1 sequences flanking the hisG insert of the disrupted locus. Transformation of strains CAI4, DDN2-3A1 or DDN3-4B1, each of genotype hwp1Δ::hisG/hwp1Δ::hisG, with this DNA effected a precise replacement of one hisG allele with URA3, generating a strain of genotype hwp1Δ::URA3/hwp1Δ::hisG. The resulting strains filamented nearly as well as the control strain (Fig. 4). Although there was a detectable reduction, which was ameliorated by uridine supplementation (Fig. 4), it was clear that the presence of the hisG repeats had a significant negative effect.

This negative effect could derive from the upstream or downstream copy of hisG individually or could be a function of their direct repetition. These alternatives were distinguished by constructing strains of genotype hwp1::hisG-URA3/hwp1::hisG, strains DDN2-3A1-A2 and DDN3-4B1-A1, and genotype hwp1::URA3-hisG/hwp1::hisG, strains DDN2-3A1-B1 and DDN3-4B1-B4, which contained a single copy of hisG upstream or downstream of URA3. Again, the insertions were placed at precisely the same position as occupied by the hisG-URA3-hisG insert. As shown in Fig. 4, insertion of either hisG-URA3 or URA3-hisG into the HWP1 locus resulted in filamentation-competent cells. The extent of filamentation

<table>
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<th>Strain</th>
<th>Arrangement of HWP1 Locus</th>
<th>Colony Morphology</th>
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<td>0.75 ± 0.09</td>
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<tr>
<td>DDN3-4B1-A1</td>
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<td>0.79 ± 0.06</td>
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</table>

*Fig. 4.* Effect of hisG on filamentation phenotype. The tested strains are indicated on the left, adjacent to an illustration of the arrangement of genetic elements at the HWP1 locus. The colony morphology of the corresponding strain after 72 h incubation at 37 °C is shown. The right-hand columns indicate the width of the hyphal fringe relative to strain CAI12 in the presence or absence of uridine.
was statistically indistinguishable from the strain containing URA3 alone, and the slight reduction relative to the wild-type control was eliminated by uridine supplementation (Fig. 4). Therefore, the direct repetition of the hisG sequences appears responsible for their negative influence on URA3 expression.

**URA3 context affects virulence**

Two prior studies had suggested that the locus of URA3 integration affected strain virulence (Cheng et al., 2003; Sundstrom et al., 2002a). Since our results indicated that the presence of hisG repeats, not the locus of integration, was more influential in determining the filamentation phenotype, we assessed how these variables affected virulence in a mouse model of disseminated disease. In agreement with our prior study, strain CAL3 (hwp1Δ::hisG/hwp1Δ::hisG-URA3-hisG) was significantly attenuated and virulence was largely restored in the rescued strain CAL5 (hwp1Δ::hisG-URA3::−485HWP1/hwp1Δ::hisG) (Fig. 5). Strain CAL10 (hwp1Δ::hisG-URA3-hwp1::115+4/hwp1Δ::hisG), identical to CAL5 at the HWP1 locus except for a frame-shift mutation in the reintroduced hwp1 allele, was equally virulent, as was strain DD27-U1 (hwp1Δ::hisG/hwp1Δ::hisG), in which the URA3 locus of the hwp1 deletion mutant was restored (Fig. 5). Therefore, as with the filamentation phenotype, the locus of insertion of the URA3 marker had no effect, since the locus differed between DD27-U1 versus CAL5 and CAL10. This conclusion was further supported by the results with strain DD27seg1-#2 (hwp1Δ::URA3/hwp1Δ::hisG). Strain DD27seg1-#2 was significantly more virulent than CAL3 (P<0-01) (Fig. 5), yet URA3 was at the identical locus and only the hisG sequences were absent. Compared to strains CAL5, CAL10 and DD27-U1, the virulence of DD27seg1-#2 was statistically different in one trial (P<0-05), but borderline significant in the second trial, suggesting that it was reduced slightly in virulence as in filamentation ability.

In contrast to our prior study (Tsuchimori et al., 2000), a reproducible and statistically significant (P<0-01) difference was observed between CAL5 and the control strain CAI12 (Fig. 5). Since CAL5 and the other strains tested were all Hwp1−, this reduced virulence might reflect the need for HWP1 (Sundstrom et al., 2002a). For this reason, strain LPR-1 was tested. LPR-1 is similar to CAL5 except for the presence of a functional allele of HWP1. Virulence of LPR-1 did not differ from that of CAL5 (Fig. 5), suggesting that HWP1 was not critical to virulence in this model.

The morphology of the candidal cells in the kidney paralleled the in vitro results. All of the mutants, except CAL3, formed abundant hyphae in vivo (results not shown).

**DISCUSSION**

These studies emanated from the unexpected observation that apparent genetic complementation of an hwp1 null mutant was not accompanied by detectable biochemical complementation. This was fortuitous. Had an alternative DNA fragment been chosen for constructing the rescued strain and HWP1 been expressed, then the genetic test of HWP1 function would have falsely appeared to be complete and valid. The lack of detectable HWP1 expression in the rescued strain, CAL5, had several tenable explanations, which were ruled out by genetic analyses. Strains CAL8, CAL9 and CAL10, like CAL5, contained an insertion at the HWP1 locus of URA3 and a reintroduced allele of HWP1. Unlike CAL5, the reintroduced alleles contained defined null mutations. Despite these mutations, these strains were phenotypically rescued, indicating that HWP1 was not required.

Defective URA3 expression was implicated as the cause of the filamentation defect. Uridine supplementation restored filamentation to strain CAL3 (hwp1::hisG/hwp1::hisG-URA3-hisG). Cheng et al. (2003) previously reported similar results for mutants containing the hisG-URA3-hisG cassette at several different loci. Whereas they noted a measurable decline in OMPase activity of the mutants, we were unable to document a similar reduction in the hwp1 mutants. Nonetheless, URA3 was clearly implicated by analysis of
spontaneous suppressors, which demonstrated that tandem duplication of the hisG-URA3 sequences suppressed the defect. Since we were unable to detect reduced OMPase activity in broth-cultured cells, this implies that URA3 activity is conditionally reduced specifically within the context of growth on agar.

Several studies have suggested that the expression of URA3 varies with its locus of integration (Cheng et al., 2003; Lay et al., 1998; Sundstrom et al., 2002a). Our results indicate that context, specifically the presence of flanking hisG repeats, rather than locus is the more significant variable. Comparison of hwp1 mutants containing URA3 or hisG-URA3-hisG at the identical position within the HWP1 locus demonstrated that filamentation and virulence were nearly wild-type with the unflanked marker. Mutants with a single copy of hisG, either upstream or downstream of URA3, were indistinguishable from those with URA3 alone. These results are entirely consistent with prior studies. In their study of hwp1 mutants, Sundstrom et al. (2002a) demonstrated that heterozygous HWP1 deletion mutants with the genotype HWP1/hwp1::hisG ENO1/eno1::URA3 were more virulent than mutants of genotype HWP1/hwp1::hisG-URA3-hisG. Aside from the locus of integration, the marker at the ENO1 locus lacked hisG repeats. Similarly, Cheng et al. (2003) compared null mutants in which the hisG-URA3-hisG cassette was integrated at the locus of interest versus the corresponding null mutant in which the Δiro1-ura3 mutation was reverted, i.e. with and without flanking hisG sequences. Those with the Δiro1-ura3 mutation reverted were more filamentous and had enhanced virulence (Cheng et al., 2003). Similarly Brand et al. (2004) demonstrated reduced URA3 expression in a number of mutants and restoration of normal expression with integration of the marker at the RPS10 locus. Again, the poorly expressed marker was within the context of flanked hisG repeats, which were not included at the RPS10 locus (Brand et al., 2004).

This study did not address whether this effect is specific to hisG repeats, nor the mechanism(s) involved. DNA repeats, particularly tandem duplications, are subject to methyl-repeats, nor the mechanism(s) involved. DNA repeats, hisG (2002a) demonstrated that heterozygous URA3 deletion mutants with the genotype HWP1/hwp1::hisG ENO1/eno1::URA3 were more virulent than mutants of genotype HWP1/hwp1::hisG-URA3-hisG. Aside from the locus of integration, the marker at the ENO1 locus lacked hisG repeats. Similarly, Cheng et al. (2003) compared null mutants in which the hisG-URA3-hisG cassette was integrated at the locus of interest versus the corresponding null mutant in which the Δiro1-ura3 mutation was reverted, i.e. with and without flanking hisG sequences. Those with the Δiro1-ura3 mutation reverted were more filamentous and had enhanced virulence (Cheng et al., 2003). Similarly Brand et al. (2004) demonstrated reduced URA3 expression in a number of mutants and restoration of normal expression with integration of the marker at the RPS10 locus. Again, the poorly expressed marker was within the context of flanked hisG repeats, which were not included at the RPS10 locus (Brand et al., 2004).

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The status of the URA3 marker altered filamentation and virulence in parallel, indicating that marker expression is compromised in vivo as in vitro and substantiating the results of Cheng et al. (2003). Consequently, we found no evidence for a role of HWP1 in disseminated disease. This contradicts prior work supporting such a role (Sundstrom et al., 2002a); the cause of this discrepancy is unclear. We found three separate hwp1 null mutants, CAL5, CAL10 and CAL12, to be equivalent in virulence to each other and to a rescued strain, LPR-1. They were, however, all slightly attenuated relative to the control strain CAI12. If the rescued strain were not fully restored for HWP1 expression in vivo, then it might be argued that there is a modest requirement for HWP1. On the other hand, our prior study found no difference between CAL5 and CAI12 (Tsuchimori et al., 2000). In comparison with the current study, this difference is due to a slight shift in the survival curves of the control strain CAI12; the CAL5 results are statistically indistinguishable (P<0.35 by log rank) between studies. In addition, a number of variables make direct comparison difficult. The study by Sundstrom et al. (2002a) used the clinical isolate SC5314 as the control, a lower inoculum for infection and half as many mice. The culture medium, which can influence the outcome (Odds et al., 2000), may also have differed (Sundstrom et al., 2002b).
The foregoing results reinforce the importance of comparing strains that are as near isogenic as possible. Comparison of the more closely isogenic Ura− strains CA14 and CA14 (hwp1Δ::hisGI/hwp1A::hisGI) would have hinted at the URA3 effects with regard to filamentation, and better strain design could have prevented the previous erroneous conclusions (Sharkey et al., 1999; Tsuchimori et al., 2000). While further highlighting the problem inherent in use of the URA3 marker, this work reinforces the conclusions of others that expression can be reliably controlled with proper strain construction (Brand et al., 2004; Cheng et al., 2003).

ACKNOWLEDGEMENTS

This work was supported by Public Health Services grant AI46249 from the National Institutes of Health. We wish to thank Abiodun Akintilo for her valuable technical contributions to this work.

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