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SHORT COMMUNICATION

Characterization of Hwp2, a *Candida albicans* putative GPI-anchored cell wall protein necessary for invasive growth

Peter Hayek, Leila Dib, Pascal Yazbeck, Berna Beyrouthy, Roy A. Khalaf*

[a]*Natural Sciences Division, Lebanese American University, P.O. Box 36, Byblos, Lebanon*

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Filamentation

Summary

Various factors are thought to be responsible for *Candida albicans* virulence, such as lipases, proteases and adhesins. Many of these factors are GPI-anchored cell surface proteins responsible for pathogenicity. Hwp2 is a putative GPI-anchored protein. The purpose of this study is to characterize the role of Hwp2 regarding filamentation on various filamentation-inducing and non-inducing solid and liquid media, virulence in a mouse model of disseminated candidiasis, and drug resistance to six widely used antifungal agents, by creating a homozygous null *hwp2* strain and comparing it with the parental and a revertant *HWP2*$^{+}$strain. It was observed that an *hwp2Δ* strain was highly filamentation-deficient on solid agar media as opposed to most liquid media tested. Furthermore, the mutant strain was slightly reduced in virulence compared to the wild strain since all mice infected with the control strain died after 6 days of injection compared with 11 days for the mutant. These results indicate a possible role for Hwp2 in adhesion and invasiveness. Finally a previously unidentified 37-amino-acid-long, stretch of Hwp2, possibly involved in protein aggregation, was found to align with high sequence identity and exclusively to *C. albicans* cell wall proteins.

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Introduction

The yeast *Candida albicans* is one of the leading fungal pathogens causing death in immunocompromised individuals. *Candida* infections range from superficial oral to deadly systemic with *C. albicans* being responsible for at least half of all candidemia cases (Warnock 2007). Depending on the environment on which it grows, *C. albicans* shifts reversibly from yeast to a true hyphal morphology including an intermediate pseudohyphal form (Brown and Gow 1999). Hyphal forms are found in many patients suffering from candidiasis and are thought

*Corresponding author. Tel.: +9547262x2216; fax: 9944851.
E-mail address: roy.khalaf@lau.edu.lb (R.A. Khalaf).

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to be necessary for infection and virulence. (Sudbery et al. 2004).

The success of *C. albicans* as a pathogen is related in part to such polymorphism as strains blocked in one of the above-mentioned morphologies proved to be non-virulent. Under *in vitro* conditions, several media and conditions can trigger *C. albicans* hyphae formation. The most powerful trigger is liquid serum at human body temperature (Ernst 2000). Synthetic media are also able to induce filamentation under defined conditions such as Lee's medium at 37 °C (Lee et al. 1975) and Medium M199 at 37 °C under basic conditions. Starvation due to carbon depletion also triggers filamentation and agar invasion on all media, including Sabouraoud agar (Cullen and Sprague 2000). In addition, *C. albicans* can form chlamydospores, thick-walled spherical cells that are produced under microaerophilic conditions combined with poor nutrients such as cornmeal agar (Montazeri and Hedrick 1984). Their physiological role is as yet unclear but they have been isolated from the lungs of AIDS patients (Chabasse et al. 1988).

Various factors are thought to be responsible for virulence, such as lipases, proteases and adhesins. Many of these factors are glycosylphosphatidylinositol (GPI)-modified-anchored cell surface antigenic determinant proteins found from lower to higher eukaryotic organisms (Eisenhaber et al. 2001). They are known either to incorporate covalently to the cell wall or to remain attached to the plasma membrane (de Groot et al. 2003). Several functions are attributed to them depending on the organism; such functions may vary from cell wall biosynthesis, to determination of surface hydrophobicity and antigenicity, in addition to adhesion and virulence (Sundstrom 2002). To date, 70 of the 115 GPI genes identified in *C. albicans* have a mutant construct (Plaine et al. 2008), and of the 15 tested for virulence 12 have shown reduced virulence compared to the wild-type strain (Richard and Plaine 2007). Possible roles of *C. albicans* GPI proteins in virulence can be attributed to the following: cell wall biosynthesis and modeling through Phr1, Phr2 and Utr2 (Richard and Plaine 2007); combating macrophage-induced oxidative stress through Sod5 (Fradin et al. 2005); specific enzymatic functions such as (i) Plb5, a phospholipase B that is necessary for *in vivo* organ colonization (Theiss et al. 2006) and (ii) Rbt1 a predicted GPI protein that is suspected to interact with and impair the immune system of the host (Braun et al. 2000); and adherence to host tissue through representatives of the Als protein family and Hwp1 (Sharkey et al. 1999; Hoyer 2001).

Hyphal Wall Protein 2 (*HWP2*) is a 908-amino-acid-long protein whose RNA transcript was detected so far only under the filamentous state in *C. albicans* and in the presence of the hyphal transcriptional activator Efg1 (Sohn et al. 2003). On the other hand, Tup1, anchored to the *HWP2* promoter by a yet unidentified DNA-binding protein, mediates repression of *HWP2* under non-hyphal-inducing conditions. Hwp2 shares conserved domains of high homology with the two above-mentioned well-characterized GPI-anchored proteins, Hwp1 and Rbt1. However, homology has so far only been reported at the C-terminus GPI anchorage site, and at the N-terminal hydrophobic region (de Groot et al. 2003).

The aim of this study was to assess the role of Hwp2 in filamentation, virulence, and drug resistance. Drug resistance was chosen since a reduction in pathogenicity might be mirrored by increased drug susceptibility. This was achieved through the generation of an *hwp2* null strain and comparing growth of the mutant to the parental and a revertant strain in various inducing and non-inducing liquid and agar media. Virulence was assessed in a mouse model of disseminated candidiasis, while antifungal susceptibility to four widely used azoles, namely fluconazole, ketoconazole, itraconazole, and voriconazole, in addition to Amphotericin B and the relatively new echinocandin, caspofungin, was determined by the *E*-test method. Finally the Hwp2 ORF was aligned with that of its two closest homologues, Hwp1 and Rbt1, in an effort to identify additional functional domains amongst the three closely related proteins.

Materials and methods

Yeast strain, media preparation and cell growth

The *C. albicans* RM1000 (*ura3Δ*::*λimm434*/*ura3Δ*::*λimm434his1*::*hisG*/*his1*::*hisG*) histidine and uridine auxotroph strain was used in this study (Negredo et al. 1997).

Cells were maintained and grown on the rich non-selective PDA (HiMedia, India) medium (Kaiser et al. 1994). For selective growth, yeast nitrogen base (YNB) synthetic medium (Fluka, Switerzland), lacking specific nutrients was utilized (Kaiser et al. 1994).

For filamentation studies on liquid media, strains were grown in the following media at both 30 °C and 37 °C for 2 (germ tube formation), 12 and 24 h (hyphal extensions): M199 with Earle's salts, L-Glutamine and 150 mM HEPES, at pH 4 or buffered

to pH 7.5 (Sigma-Aldrich, Germany), Lee's media buffered to pH 6.8 (Lee et al. 1975), and fetal bovine serum (FBS, Biowhittaker, Belgium). For solid media invasive growth, strains were grown on Sabouraud agar+dextrose (Oxoid, UK) for 14 days at 30 and 37 °C and monitored daily for filamentous growth, and on yeast nitrogen base (Sigma-Aldrich, Germany) supplemented with 0.1% dexrose. Filamentous colonies were washed with tap water to assess invasive growth. Hyphal formation on cornmeal agar supplemented with 1% Tween 80 was prepared according to the manufacturer's instructions (HiMedia). Briefly, media were inoculated with a cut streak and covered with a glass cover slip as described previously (Buckley et al. 1982), incubated at 28 °C and monitored daily for 5 days.

Images were generated utilizing an Olympus E330-ADU-1.2x stereomicroscope for colony morphology and an Olympus CX41 for cell imaging coupled with a Sony DSC-S40 digital camera.

Strain construction

The homozygous *Δhwp2* strain was generated by transforming strain RM 1000 with hybrid *URA3-HWP2* and *HIS1-HWP2* deletion fragments that contained the functional *URA3* and *HIS1* alleles, respectively, flanked by 100 bp of the *HWP2* allele as described previously (Khalaf and Zitomer 2001; Gola et al. 2003; Dib et al. 2008). Verification of correct integration was confirmed by PCR analysis. Four sets of primers were utilized. The first set involved *HWP2* internal primers to confirm the presence or absence of an *HWP2* allele. The second and third set involved a primer that hybridized outside the *HWP2* integration site and either an internal *URA3* or an *HIS*1 primer. Finally *ACT1* primers were used as a control for the quality of DNA preparations. To generate a revertant *ura3*::*HWP2*/*hwp2*::*HIS1* strain, the *hwp2* null strain was transformed with a DNA cassette containing the entire *HWP2* ORF from −300 to +2808. Ura^- transformants were counter-selected for on yeast nitrogen base media supplemented with 5-fluoroorotic acid (5-FOA) (0.17% YNB, 0.5% ammonium sulphate, 0.005% uridine, 0.1% 5-FOA, 2% dextrose, 2% agar). Integration of a functional *HWP2* allele was verified by PCR amplification utilizing *HWP2* internal primers.

DNA extraction

DNA was extracted from all strains prior to PCR analyses as described previously (Khalaf and Zitomer 2001).

Yeast transformation

C. albicans strain RM1000 was transformed with the *URA3-HWP2* fragment, the *HIS1-HWP2* fragment, or plasmid pABSK2 for the wild-type control, using a modification of the Alkali Cation Yeast Transformation Kit (QBiogene, Canada) as described previously (Khalaf and Zitomer 2001).

Murine model of disseminated candidiasis

Four- to six-week-old BALB/c mice (Lebanese American University stock) weighing 20–30 g were injected via the lateral tail vein with 200 μl of 5×10^6 cells suspended in PBS solution, pH 7.5. Six mice were injected/strain tested. Mice were given food and water ad libitum and monitored 3 times daily for survival, over a period of 30 days.

Antifungal susceptibility assay

The antifungal susceptibility test was performed on RPMI 1640 medium (with L-glutamine and 165 mM MOPS, Biowhittaker) using the Epsilometer test (*E*-test) method. *E*-test strips of fluconazole (FL), voriconazole (VO), ketoconazole (KE), itraconazole (IT), amphotericin B (AP), and caspofungin (CS) were supplied by AB Biodisk. Inoculum preparation, inoculation, and strip application were performed according to the manufacturer's instructions. Minimal inhibitory concentration (MIC) readings were done after 24-h incubation at 35 °C. The MIC interpretive breakpoint criteria utilized have been previously determined (Rex et al. 1997; Clancy and Nguyen 1999; Pfaller et al. 2003; Therese et al. 2006; Pfaller et al. 2006). The *C. albicans* CLSI quality control strain ATCC 90028 was utilized as a reference strain with the MIC values obtained falling within the control limits.

Homology assessment

Regions of sequence similarity were identified by the NCBI alignment tool blast2seq at http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi. Amino acid sequences were aligned using the CLUSTALW 2.0 program available at www.ebi.ac.uk/clustalw/. The Interproscan EBI tool available at www.ebi.ac.uk/InterProScan was utilized to determine whether a homologous sequence is part of a known conserved protein domain or motif. Finally, to predict the three-dimensional structure of the newly identified regions, sequences were run against the PDB protein database at www.pdb.org.

Statistical analysis

The Kaplan–Meier survival curve and the log Rank test were performed on the data collected using the SPSS computer software (SPSS Inc., USA) to assess the significance of any discrepancy.

Results

Filamentation on liquid media

Strains were incubated in Lee's, M199 pH4 and 7.5 and FBS, at 30 and 37 °C, and monitored at 2, 12 and 24 h intervals. No significant differences in hyphal induction time or filamentation levels were observed under all media tested. However, in the *hwp2* null strain a slightly lower percentage of cells responded to serum induction compared to the wild type.

Filamentation on solid media

The filamentation ability of the *hwp2* null mutant, the heterozygote, revertant, and wild-type strains was investigated by streaking on Sabouraud agar and incubating at 30 and 37 °C for 14 days. In addition, filamentation and chlamydospore formation were examined by growth on cornmeal agar+Tween 80 for 5 days at 28 °C as previously described. On Sabouraud agar the *hwp2* null mutant was not able to filament while all the remaining *HWP2*$^+$ strains showed clear filamentation abilities (see Figure 1). A similar pattern was observed on YNB +0.1% dextrose media, with the mutant forming shorter and much less abundant filaments, but this difference required 5 days instead of 14 to manifest itself (data not shown). The null mutant was also deficient in hyphal formation on cornmeal agar (Figure 2).

Incubating the mutant strain under 5% physiological CO_2 concentrations, a potent hyphal inducer (Klengel et al. 2005) did not rescue the phenotype. To investigate whether the filaments formed invaded agar or were formed on the surface, we attempted to wash away all agar filamenting colonies with tap water with no success while the mostly non-filamentous *hwp2* null strain was washed off the plate (data not shown).

Murine model of disseminated infection

Using the mouse systemic candidiasis model, it was observed that all mice, regardless of strain utilized, died within 11 days of infection. However, mice infected with the heterozygote and null

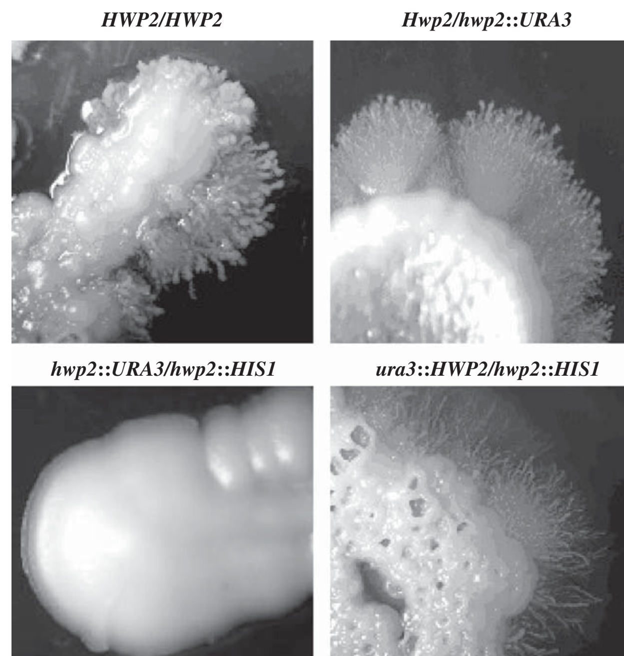

Figure 1. Growth on Sabouraud agar. The wild-type, heterozygote, revertant, and *hwp2* null strains were streaked on Sabouraud dextrose agar for 14 days at 30 °C. Colony edges were photographed at low magnification (20 ×) to show the presence of hyphal extensions. Note the lack of filamentation in the *hwp2* null strain.

mutant strains survived longer because by day 6, all of the mice infected with the wild-type and revertant strains died, while both the *HWP2/hwp2* heterozygous strain and the *hwp2/hwp2* homozygous deletion strain had 83% and 67% survival rates (Figure 3). The discrepancy between the wild-type and null mutant was not statistically significant ($p = 0.09$).

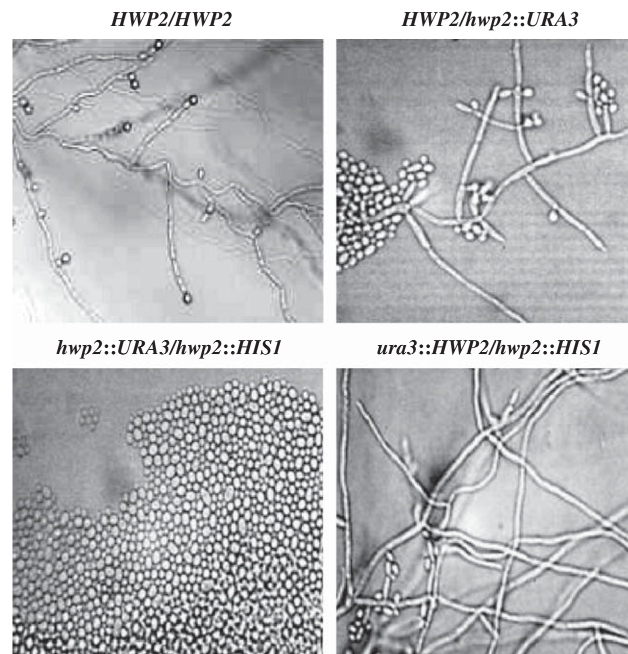

Figure 2. Growth on cornmeal agar media. The wild-type, heterozygote and *hwp2* null mutant were grown under microaerophilic conditions on cornmeal agar for 5 days at 28 °C. Note the extensive network of filaments in the *HWP2*$^{+}$ strain with chlamydospore formation and the lack of filamentation in the null mutant. Cells were photographed at $1000\times$ magnification.

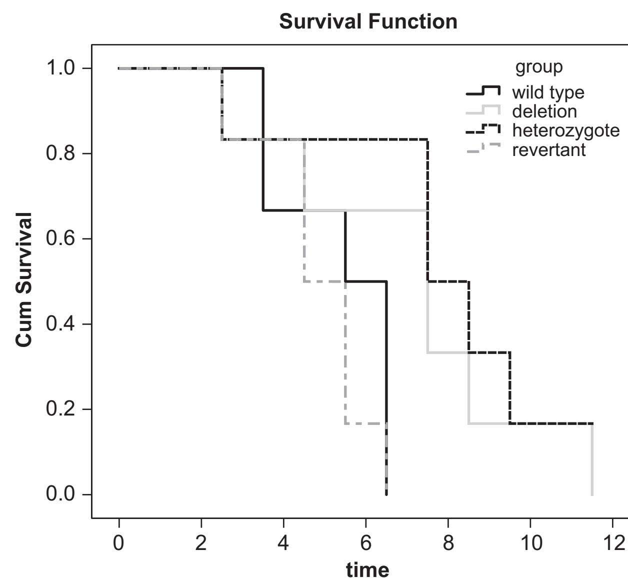

Figure 3. Murine model of disseminated candidiasis. The percentage survival of mice following intravenous injection of 6×10^{6} cells of the *HWP2/HWP2*, *HWP2/hwp2* and *hwp2/hwp2* strains. Six mice were injected/strain. Note that the *hwp2* null and *HWP2/hwp2* strains remained pathogenic although slightly attenuated in virulence compared to the wild type. Discrepancies between the wild-type and null mutant were not.

Table 1. Antifungal susceptibility table

	RM1000+pABSK2	*HWP2/hwp2*	*hwp2/hwp2*	*ura3::HWP2/hwp2::HIS1*
Amphotericin B	0.19	0.25	0.19	0.19
Caspofungin	0.032	0.094	0.094	0.094
Fluconazole	1.0	1.0	0.75	1.0
Ketoconazole	0.016	0.016	0.023	0.016
Itraconazole	0.023	0.032	0.032	0.032
Voriconazole	0.016	0.023	0.016	0.032

The antifungal drug susceptibility of the null *hwp2* mutant was determined and compared with that of the wild type and heterozygote strains using the *E*-test method. As can be seen no significant discrepancy was noted for any drug tested. Numbers represent MIC's in μg/ml. Experiment was repeated three times. Values represent a typical experiment. Discrepancies were not statistically significant ($p > 0.05$).

Antifungal susceptibility

The *E*-test was performed to determine strain sensitivity to amphotericin B, four azoles, and caspofungin on RPMI 1640+165 mM MOPS. After 24 h of incubation, the MIC values were read at the lowest drug concentration at which there was complete inhibition of growth. The MIC values were determined for RM1000+pABSK2, *HWP2/hwp2*, *hwp2/hwp2* and the *ura3::HWP2/hwp2::HIS1* revertant. As can be seen in Table 1, no significant discrepancy amongst the three strains was noted for all six drugs tested ($p < 0.05$).

Homology assessment

The ORF of Hwp2 was aligned with it two closest homologues, Hwp1 and Rbt1, in an effort to determine possible regions of similarity outside the GPI anchor and the N-terminal hydrophobic region using the NCBI alignment tool blast2seq and the CLUSTALW 2.0 software. A highly conserved 37-amino-acid-long sequence was found twice in Hwp2. This sequence was found to align with high sequence identity to two distinct sequences in Hwp1 and Rbt1. Further analysis of this sequence revealed a 68% polar amino acid composition (Figure 4).

```
Hwp1   313  VVTVTSCSNSVCTESEVTTGVIVITSKDTIYTTYCPL
Hwp1   378  VITVTSCSESSCTESEVTTGVVVVTSEETVYTTFCPL
Hwp2   711  VITVTSCSDNLCSKTEVTTGVTVITSDTTSYTTYCPL
Rbt1   515  VITVTSCSNNACSKTEVTTGVVVVTSEDTIYTTFCPL
Hwp2   222  VVTLTSCSNNACSQSEITTGAIVVTDKETVYTTYCPL
Rbt1   417  IVTITSCSNNACSESKVTTGVVVVTSEDTVYTTFCPL
            ::*:****:.  *:::::***.  *:*..  *  ***:***
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Figure 4. Multiple sequence alignment. The 37 amino-acid-long regions of similarity amongst Hwp1, Rbt1 and Hwp2 were aligned via CLUSTALW 2.0 software and analyzed for the relative ratio of polar to non-polar residues. As can be seen, around 68% of the residues are polar, which suggests localization outside the core protein. Red residues refer to non-polar amino acids, green refers to polar neutral amino acids, blue residues are acidic, while pink denotes basic residues. *Refers to identical amino acids, to highly conserved substitutions and to conserved substitutions. All sequences were composed of at least 60% identical and 80% conserved amino acids compared with the Hwp2 sequence from amino acids 222–258.

Discussion

Focus on *Candida albicans* cell wall proteins in general and on GPI anchor proteins in particular is on the increase as many of these proteins are antigenic determinants that have been shown to play a role in virulence and filamentation, and are thus possible targets for novel antifungal agents. The reason behind characterizing Hwp2 was based on the fact that its two closest homologues, Hwp1, necessary for adhesion in biofilm formation (Nobile et al. 2006), and Rbt1, required for virulence (Braun et al. 2000), have both been previously characterized. We chose to generate our deletion utilizing non-recyclable marker cassettes instead of the classical urablaster approach since Sharkey et al. (2005) showed that flanking *hisG* repeats can cause loss of ura expression, generating a filamentation and virulence-attenuated phenotype that can be misinterpreted as a mutant phenotype. In our study, all liquid and solid media experiments utilized for phenotype comparisons were performed in duplicates: one set supplemented with 75 μg/ml uridine and histidine and the other unsupplemented. No discrepancy in phenotypes was present between the two conditions.

Hwp2 does not seem to play a major role in filamentation in liquid media even though the mutant strain did show a slight decrease in hyphal formation in serum. Sohn et al (2003) previously showed that *HWP2* is activated by Efg1 in media containing serum, thus it is not surprising that the *hwp2Δ* phenotype was different from that of the parental. Expression of *HWP2* in Lee's and M199 pH 7.5 has not been

investigated and the possibility that *HWP2* is not expressed under these conditions cannot be ruled out, resulting in no morphological difference.

The most dramatic morphological difference between the mutant and wild type was observed on solid media, mainly as a lack of invasive growth. Glucose starvation is a fungal-wide filamentation signal since it has been previously shown to be responsible for filamentation of *Saccharomyces cerevisiae* regardless of nitrogen availability (Cullen and Sprague 2000). In *C. albicans* several factors repress filamentation including availability and type of carbon sources (Hayes 1966). In our case, dextrose depletion after 14 days of growth might have acted as a positive trigger for filamentation in the wild type. Under such conditions Hwp2 expression mediates invasion. To confirm our findings, strains were grown on glucose-depleted medium (YNB+0.1% glucose). Discrepancies in filamentation were also observed – even though the mutant exhibited a slight filamentation potential – but only after 5 days of growth, confirming the role of glucose in filamentation. Extending our observations to 14 days confirmed the fact that the phenotype is due to a defect in and not merely a delay of filamentation. One of the requirements for formation of filaments and invasion is the ability of yeast cells to change its cell surface composition, enabling the cells to adhere to each other and to invade the agar substratum (Cullen and Sprague 2002). Adherence is essential for the ability of yeast *C. albicans* to form filaments and invade agar. Relationship between adherence and hyphae formation in *C. albicans* has been previously shown with the surface protein Int1. An *int1* null strain is unable to produce hyphae and is non-adherent to epithelial cells (Gale et al. 1998). The effects observed on cornmeal agar were similar to that on Sabouraud and YNB, with invasion being limited to the *HWP2*$^+$ strains after 5 days of incubation. Cornmeal agar is inherently glucose poor and thus any phenotypic difference amongst strains does not require 2 weeks to manifest itself. The *S. cerevisiae* Hwp2 homologue is Flo11/Muc1 (www.candidagenome.org). Muc1 is a mucin-like membrane-bound glycoprotein that modulates cell adhesion and is required for diploid pseudohyphal formation and haploid invasive growth. Wild-type *S. cerevisiae* strains grow invasively forming pseudohyphae on starch medium, a phenotype abolished in a *muc1* mutant (Lambrechts et al. 1996). Compared to the *HWP2*$^+$ strains, our mutant was also unable to filament on high starch containing cornmeal agar medium.

Even though the *hwp2* null strain could not invade agar, it was still virulent, implying the ability to invade tissue *in vivo*. Since *C. albicans* is known to have multiple pathways for virulence to account for its success as a pathogen compensation for the absence of Hwp2 on the cell wall might have occurred to insure the survival of the organism. Furthermore, the null mutant showed no reduction in drug susceptibility to 6 commonly used antifungal agents. In fact, a 3-fold increase in MIC of caspofungin, a cell-wall-destabilizing compound (Letscher-Bru & Herbrecht 2003), was noted for the mutant strain that lacks Hwp2, a cell wall component. However, the increase did not reach statistical significance and it was still far below the breakpoint value for resistance.

Multiple sequence alignment of Hwp2 with Rbt1 and Hwp1 revealed a 37-amino-acid-long region of similarity (greater that 60% identity) found twice in each protein and not reported previously. In fact, this sequence was present in no less than 10 cell wall and GPI-anchored *C. albicans* proteins with a 50% or greater identity and not detected in non-cell-wall proteins. Analysis of this sequence revealed a two-thirds polar amino acid composition, suggesting localization outside the core of the protein, and thus possibly involved in host–pathogen interaction. NCBI and Interproscan at EBI databases could not identify the sequence as a conserved domain. Similarly, no 3D structure was attributed to it at the PDB database. Interestingly, the Als family of cell wall adhesins has serval-thr-rich sequences. Als9 has an IIVVTT sequence with a 96% aggregation potential according to the TANGO polypeptide aggregation program (Otoo et al. 2008, P. Lipke, personal communication). This sequence has high similarity to the AIVVT sequence of Hwp2 identified in this study and which aligns with high homology to similar sequences in Rbt1 and Hwp1 (Figure 4). In adhesins, such repeats are usually found in the mid-region, sandwiched in between the N-terminal globular Ig-like domain and the C terminal glycosylated stalk region that extends the active site from the cell wall surface. Since protein aggregation is a possible precursor to adhesion, this 37-amino-acid-long sequence might be a key sequence involved in adhesion and invasive growth. Its presence in several GPI-anchored proteins and the little information available open the way for further studies of the domain.

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