Expression and Interstrain Variability of the YPS3 Gene of\nHistoplasma capsulatum\n
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The YPS3 locus of the dimorphic fungus Histoplasma capsulatum encodes a secreted and surface-localized protein specific to the pathogenic yeast phase. In this study we examined this locus in 32 H. capsulatum strains and variants. Although protein production is limited to a select group of strains, the North American restriction fragment length polymorphism class 2/NAm 2 isolates, the locus was present in all the strains we examined. The YPS3 gene is well conserved in its 5′ and 3′ regions but displays an intragenic hypervariable region of tandem repeats that fluctuates in size between strains. This feature is similar to that seen with genes encoding several cell surface proteins in other fungi.

Histoplasma capsulatum is a dimorphic fungal pathogen that is the causative agent of histoplasmosis, a respiratory and systemic disease of particular danger to the immunocompromised. Essential to the pathogenicity of H. capsulatum is the ability to transition from the mycelial to the pathogenic yeast phase of growth, a transformation that happens after the shift from ambient to human body temperature (19, 20). Associated with this conversion is alteration in the expression of many genes; nearly 500 phase-regulated genes have been identified to date (4, 7, 9, 11, 12, 17, 18, 22, 25). Genes that are upregulated during the pathogenic yeast phase of growth have been of particular interest to researchers. Two yeast phase-upregulated genes, CBP1 and AGS1, have been implicated in pathogenesis, while the functions of others remain unknown (11, 17, 24).

The YPS3 gene of Histoplasma capsulatum was first identified in 1989 by Keath et al. in a differential hybridization screening as one of five identified transcripts unique to the yeast phase of growth (17). It encodes a 20 kDa yeast phase-specific protein that is both secreted and surface localized. Yps3p localizes to the cell surface via a mechanism of secretion from the cell and binding back to the exposed cell wall polysaccharide chitin (1). It was initially described as having variable expression in different strains of H. capsulatum. Specifically, a survey of three strains typed by restriction fragment length polymorphism (RFLP) suggested that protein production during the yeast phase was restricted to the North American class 2 strains, the most thermotolerant and virulent strains of H. capsulatum identified so far (17). In this report we sought to more thoroughly examine H. capsulatum isolates in light of the recent classification schemes that have been developed (13, 14, 16) and to better understand interstrain diversity.

MATERIALS AND METHODS

Fungal strains and culture conditions. Table 1 lists the isolates and variants used in this study. Strain G184AS ura5-11, a uracil auxotroph derived by UV mutagenesis, has been described previously (28). H. capsulatum was grown in Histoplasma-macrophage medium broth, a rich defined medium (29), in a 5% CO2–95% air atmosphere. All experiments were done with H. capsulatum grown as yeast cells at 37°C.

Bacterial strain. Plasmids were cloned and propagated in the Escherichia coli strain JM109 (F’ trnD36 proAB lacIq Δ(lacZ)M15Δ(lac-proAB) glnV44 cI4– gatC46 recA1 relA1 endA1 thi hsdR17). N-terminal sequencing. We collected Yps3p by the use of a previously described polysaccharide binding assay (1). Briefly, we washed 50 mg of crab shell chitin (Sigma Aldrich, Saint Louis, MO) once with phosphate-buffered saline (PBS) and incubated it for 1 h with filtered, concentrated supernatant from mid-logarithmic-phase G184B cultures. Following incubation, we removed the supernatant and boiled the chitin in 2× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis loading buffer (50 mM Tris-Cl containing 2% SDS, 100 mM dithiothreitol, 10% glycerol, and 0.1% bromophenol blue) for 5 min at 95°C, electrophoresed the mixture in an SDS–12% polyacrylamide gel, and electroblotted the gel onto a polyvinylidene difluoride membrane. After transfer, the polyvinylidene difluoride membrane was stained with Coomassie blue, and the band corresponding to Yps3p was excised and submitted to the Protein Facility of the Iowa State University Office of Biotechnology for N-terminal sequencing on an Applied Biosystems Model 494 Procise protein-peptide sequencing system (Applied Biosystems, Foster City, CA).

Amplification, cloning, and sequencing of the YPS3 locus. Primers were designed to amplify the open reading frame (ORF) by the use of MacVector sequence analysis software (Accelrys, San Diego, CA). We designed ORF-specific primers utilizing available sequences of the YPS3 locus from two sources. Our laboratory has previously sequenced the locus from several strains, including G182B, Downs, G184AS, G184AR, and RV26821. The Histoplasma capsulatum genome project pages (http://www.genome.wustl.edu/genome.cgi?GENOME=Histoplasma%20capsulatum; http://www.broad.mit.edu/annotation/genome/histoplasma_capsulatum/Home.html) present sequences of strains of RFLP classes 1, 2, and 3. The primers used to amplify class 2 strains were ORF upstream (5'-ATGCTGTAACATCAAATCGATCTC-3') and ORF downstream (5'-TTATGCTCCGCAGTTTATAAACG-3'). The primers used to amplify class 1 and 3 strains were ORF upstream and C1/C3 downstream (5'-TTATG CCTCCGACTTGTGATGAAAGC-3'). All genes were amplified using Triplemaster high-fidelity polymerase (Eppendorf, Westbury, NY). After amplification, we cloned these ORFs into the vector pGEM-T Easy (Promega, Madison, WI) and sequenced two to three clones by the use of BigDye Terminator v. 3.1 mix and an Applied Biosystems 3730d automated DNA sequencing instrument. Because of the nature of cloning a repetitive element into E. coli, all sequenced ORF sizes were verified by comparison to genomic DNA PCR amplifications.

Protein expression in G184AS ura5-11. To test the coding capacity of YPS3 from representative class 1 and class 3 strains, we cloned the ORFs from strains G186B and Downs into an expression vector based on the previously described telomeric shuttle plasmid pWU45 (23). This vector contains the Podospora anserina URA4 gene and a telomeric cassette for selection and maintenance in Histoplasma.

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When we used BLAST tools to examine virulence that does not produce the Yps3p protein, we revealed that the RFLP class 1 strain Downs, a strain with relatively low conservation and size of the gene, was isolated from clinical samples. We observed that it has a larger predicted gene size also. To determine the conservation and size of the gene on a broader scale, we examined conserved differences in YPS3 gene.

### Table 1. Analysis of the YPS3 gene in 32 isolates

| Strain name | Source and/or description | Classification method | Analysis method | ORF size (bp) | Yps3p expression |
|-------------|--------------------------|-----------------------|----------------|---------------|-----------------|
| UWclin01    | Clinical isolate, Wisconsin | RFLP | seq WB | 691 | – |
| Downs (ATCC 38904) | Clinical isolate, 1968, Missouri | Other | seq PC, WB | 600 | – |
| UCLA 531S   | Clinical isolate, California | RFLP | seq WB | 675 | – |
| W#1         | AIDS patient, 1992 | seq | &lt; WB | 675 | – |
| CDC5568     | Virginia clinical isolate | seq | &lt; WB | 600 | – |
| W#2         | Clinical isolate, AIDS patient, 1992 | PCR | WB | 415 | + |
| W#3         | Clinical isolate, AIDS patient, 1991 | PCR | WB | 415 | + |
| W#6         | Clinical isolate, AIDS patient, 1992 | PCR | WB | 415 | + |
| W#9         | Clinical isolate, AIDS patient, 1989 | PCR | WB | 415 | + |
| CDC5563     | Virginia soil isolate | PCR | WB | 415 | + |
| CDC5564     | Virginia soil isolate | PCR | WB | 415 | + |
| CDC5565     | Virginia soil isolate | PCR | WB | 415 | + |
| G217B (ATCC 26032) | Clinical isolate, Louisiana | PCR | WB | 414 | + |
| G217A (ATCC 26031) | Clinical isolate, Louisiana | NAm | seq WB | 414 | + |
| G222B (ATCC 26034) | Clinical isolate, Louisiana | seq | &lt; WB | 567 | NT |
| UWclin02    | Clinical isolate, Wisconsin | H1 | seq FC, WB | 552 | – |
| G184AS      | Variant of G184AR, lacks alpha-(1,3)-glucan | H1 | seq FC | 552 | – |
| G186AR (ATCC 26027) | Clinical isolate, Panama | H1 | seq WB | 552 | – |
| G186B (ATCC 26030) | Clinical isolate, Panama | H1 | PCR WB | 552 | – |
| G186AS      | Variant of G186AR, lacks alpha-(1,3)-glucan | H1 | PCR WB | 552 | – |
| G186AR (ATCC 26029) | Clinical isolate, Panama | H1 | PCR WB | 552 | – |
| G186AE      | Variant of G186AR, lacks alpha-(1,3)-glucan | H1 | PCR WB | 552 | – |
| JHMA(H502)  | Clinical isolate, Sao Paulo, Brazil | H1 | PCR WB | 552 | – |
| ERS(H201)   | Clinical isolate, Sao Paulo, Brazil | H1 | PCR WB | 552 | – |
| CFLA(H285)  | Clinical isolate, Sao Paulo, Brazil | H1 | PCR WB | 552 | – |
| MSL(H1)     | Clinical isolate, Sao Paulo, Brazil | PCR WB | 552 | – |
| MFG(H381)   | Clinical isolate, Sao Paulo, Brazil | PCR WB | 552 | – |
| 22-0453     | Clinical isolate, AIDS patient, 1992 | PCR WB | 552 | – |
| RV26821R (ATCC 32281) | Clinical isolate variant, Belgium | PCR WB | 552 | – |
| UWclin03    | Clinical isolate, Wisconsin | PCR WB | 552 | – |
| W#13        | Infected bats, Houston Zoo | LaM A | seq WB | 612 | – |
| W#10        | Clinical isolate, AIDS patient, 1989 | PCR WB | 415 | + |

*Classification are from references 6 and 7.

G. capsulatum and the ampicillin resistance gene for selection in Eichnerichia coli. Expression in this vector is achieved via a fusion of the YPS3 ORF with the promoter from the H2B gene, which encodes the constitutively expressed histone H2B.

**Protein detection.** For Western blot analysis, samples were denatured by incubation in 50 mM Tris-Cl containing 2% SDS, 100 mM dithiothreitol, 10% glycerol, and 0.1% bromophenol blue for 5 min at 95°C, electrophoresed in an SDS-12% polyacrylamide gel, and electroblotted onto a nitrocellulose membrane. This membrane was blocked for 1 h with Tris-buffered saline (25 mM Tris, 100 mM NaCl, 2.7 mM KCl) containing 0.1% SDS, 0.05% Tween 20, and 5% dried milk. The blot was then incubated with a 1:10,000 dilution of Yps3p-specific antigen (H1). Nonspecific antibody was removed by three washings for 20 min per washing in 0.01% SDS-0.05% Tween 20-Tris-buffered saline. The washed nitrocellulose membrane was incubated in a 1:6000 dilution of horse-radish peroxidase-labeled goat anti-rabbit antibody (Bio-Rad, Hercules, CA).

**Immunofluorescence microscopy.** H. capsulatum yeast cells from late-log-phase Histoplasma-macrophage medium broth cultures were washed in PBS and stained indirectly using a 1:10,000 dilution of Yps3p-specific rabbit anti-serum followed by a 1:100 dilution of goat anti-rabbit immunoglobulin G Alexa Fluor 488 (Invitrogen, Carlsbad, CA) for 20 min at 4°C. Stained cells were washed with PBS, and a 10-μl aliquot was spread on a prewashed glass slide, air dried, and mounted under a glass coverslip using Prolong Gold antifade reagent (Invitrogen). Images were collected with an AxioCam black-and-white charge-coupled device camera, and the images were analyzed using Open-Label 5.0.2 software (Improvision, Lexington, MA).

**RESULTS**

**Analysis of the YPS3 locus in 32 strains.** Early analyses revealed that the RFLP class 1 strain Downs, a strain with relatively low virulence that does not produce the Yps3p protein, has a YPS3 gene that appears larger than the expressed gene in the class 2 strain G217B (15). Similarly, when we used BLAST tools to examine the sequenced class 3 strain G186AR, we observed that it has a larger predicted gene size also. To determine the conservation and size of the gene on a broader scale, we examined the ORF and tested for Yps3p protein expression in 32 strains and variants by use of a variety of techniques (Table 1). These strains were isolated from clinical samples or the environment, and some strains have previously been classified based on RFLP class or DNA sequencing of individual genes (13, 14, 16). All 32 strains we examined had the YPS3 gene. We noted well-conserved differences in gene size between phylogenic groups. Additionally, we observed a strong correlation between a gene size of approximately 414 bp and production of the Yps3p protein (Table 1).

**Individual isolate sequencing.** We designed ORF-specific primers based on available sequence data from the Histoplasma capsulatum genome and amplified the locus from isolates selected from Table 1. The selected strains were representative of three described RFLP classes as well as unclassified clinical isolates and strains from the variant Histoplasma capsulatum var. duboisi, typically associated with African histoplasmosis. After amplification, we cloned and sequenced the ORF. The ORF appears well conserved in the 5′ and 3′ regions. This conservation includes 100% predicted amino acid identity in the first 24 amino acids, a region that, according to our N-terminal sequencing results, encodes a secretion signal sequence that is cleaved from the mature protein after the first proline (MLNIKISSSTLLLASSSLVAAAR P/GEYPTD...), where the italicized residues correspond to the
signal sequence). In all strains examined, the gene size differences among strains are due to a region of intragenic variability that starts approximately 100 bp after the ATG start codon (Fig. 1) and accounts for 7% to 43% of the gene's length.

Analysis of the intragenic variable region. Using sequence analysis software, we analyzed the DNA and predicted amino acid sequences of the sequenced strains. The insertions in all of the strains sequenced resulted in an in-frame predicted protein, three examples of which are shown in Fig. 2A. The intragenic variability is composed of a series of between 2 and 20 well-conserved amino acid repeats with the consensus sequence YPTDK. In strains with multiple copies of the repeat,
18% to 40% of the repeats showed some variability at the first, third, or fourth position in the form (Y,H)P(I,T)(D,H),K (Fig. 2). Two copies of the repeat in the sequenced class 1 strains also have the alternate form YPVDDK. The observed intragenic variability occurs at the same point where Yps3p diverges from its homolog Bad1, the well-described Blastomyces dermatitidis Blastomyces dermatitidis adhesin, immunomodulatory agent, and virulence factor (1–3). The heterologous region of BAD1 also involves a repetitive element of a longer 24-amino-acid length but comprised of similar amino acids (10). The RFLP class 2 strains, which express the Yps3 protein, show the smallest number of repeats, averaging two. The analyzed RFLP class 3 strains have 11 or 12 copies of the repeat. The RFLP class 1 strains have 18 to 20 copies of the repeat, while strains of the African subtype H. capsulatum var. duboisii have 16 to 17 copies. The differing numbers of tandem repeats between strains of the same and different RFLP classes suggest plasticity in the locus (Fig. 2B).

**Heterologous expression of the YPS3 ORF.** Whereas concentrated culture supernatants of all class 2 strains tested contained Yps3p, we could not detect Yps3p produced by a panel of class 1 or 3 strains tested on the cell surface (Table 1) or released extracellularly (reference 1 and data not shown). It was previously hypothesized that this intragenic variability could possibly result in protein instability, a potential explanation for why class 1 and class 3 strains do not produce Yps3p (15). To address this, we expressed the cloned ORFs from the class 1 strain Downs, the class 2 strain G217B, and the class 3 strain G186B in the class 3 uracil auxotroph G184AS ura5-11 under the control of the heterologous promoter from the Histoplasma capsulatum histone H2B gene.

FIG. 2. The top panel (A) shows three predicted amino acid sequences with variable-length repeats. Figure 2B shows a scaled diagram of the Yps3p protein from several strains of various classifications. The black bars indicate the well-conserved N and C termini of the gene. Each hexagon represents a tandem repeat element. Gray-shaded hexagons are repeats of the consensus YPTDK sequence. White hexagons indicate a tandem repeat with one or two substitutions or an insertion, as described in the text. H. duboisii, Histoplasma capsulatum var. duboisii.
revealed that under heterologous control the Yps3p protein from class 1 or class 3 strains can be produced in a class 3 strain, is reactive with our class 2 Yps3p antibody, and is predictably larger (Fig. 3A). The heterologously expressed class 1 (Fig. 3B) or class 3 (data not shown) protein is also surface localized, which is also a characteristic of natively expressed Yps3p in class 2 strains (1), although expression in general in these strains occurs at very low levels.

DISCUSSION

These findings demonstrate marked interstrain diversity in the selectively expressed YPS3 locus of the thermally dimorphic fungal pathogen Histoplasma capsulatum. They reveal a hypervariable region that encodes a 5- to 6-amino-acid tandem repeat element. There is evidence of plasticity in this repetitive element, with strains containing between 2 and 20 copies. The presence of a larger-sized insert does not prevent production of protein when it is expressed during yeast-phase growth under the control of a heterologous promoter. While this study used yeast-phase thermal growth conditions, it was an in vitro study, and future directions of research include a detailed analysis of protein production during infection in all classes of growth. Although patients infected with class 1 strains do not generate antibody to Yps3p, suggesting that it is also not produced in vivo (27), it is important to better understand the

FIG. 3. A. Western blotting of Histoplasma capsulatum G184AS ura5-11 yeast cells expressing the YPS3 ORF of Downs, G217B, or G186B strains by use of a heterologous H2B promoter reveals Yps3p proteins of variable sizes. The increased size of the class 1 and class 3 proteins is consistent with the larger-sized ORF due to the region of intragenic variability. B. Yps3p produced from the heterologously expressed Downs strain ORF is detectable on the surface of G184AS ura5-11 yeast cells. Left panel, immunofluorescence; right panel, differential interference contrast.

FIG. 4. Predicted protein alignment of several putative Aspergillus species homologs. These proteins share a predicted N-terminal signal sequence and a C-terminal EGF-like domain, including the conserved spacing of six cysteines (boxed) predicted to be important for disulfide bonding. The Aspergillus species are the most minimal homologs identified and lack an intragenic tandem repeat domain. The H. capsulatum strain G217B Yps3p and B. dermatitidis Bad1p homologs are shown for comparison. The large tandem repeat region of Bad1p is indicated.
regulation of this protein and whether host signals, in addition to temperature, play a role.

Intragenic repeats have recently been analyzed for several cell wall or surface proteins and have been shown to play a role in adhesive properties of these proteins. A study by Verstrepen et al. analyzed Saccharomyces cerevisiae open reading frames and identified 44 ORFs with intragenic repeats, 23 of which encode proteins with predicted cell surface localizations (26). In vitro analysis of one of these genes, FLO1, which encodes a predicted adhesin, revealed that altering the number of repeats led to differences in flocculation and adherence to polystyrene whereas longer regions of repeats increased both phenotypes (26). In addition to the Verstrepen study, intragenic repeats have been analyzed in the Candida albicans ALS3 gene, where adhesive function is also linked to more copies of a tandem repeat sequence, this one 108 bp long (21). While the results regarding tandem repeats could suggest a role for Yps3p as an adhesin, the class 2 strains that routinely produce the protein have the most minimal tandem repeat subunit (only two copies). Additionally, Far Western blotting, yeast two-hybrid, and immunofluorescence analysis of host cells probed with Yps3p have not yielded evidence for an adhesive function (data not shown).

The intragenic tandem repeat region also marks the main area of divergence from Blastomyces dermatitidis BADI. While the BAd1 protein shares a conserved N-terminal secretion signal sequence and C-terminal epidermal growth factor (EGF)-like domain with Yps3p, the intragenic repeat region is comprised of 30 copies of a longer, 24-amino-acid repeat. The Klein group has extensively characterized BAd1p and determined that it is an essential virulence factor of Blastomyces dermatitidis (3). The immunomodulatory action contributing to this virulence effect has been localized to the repeat region (5, 6). Analysis of interstrain variability in BAd1 has not yet been performed. We are currently using RNA interference to analyze the role of Yps3p during infection and to determine how the divergent intragenic regions in these two homologs might differently affect fungal virulence.

Beyond BAd1, we have recently analyzed the genomes of several Aspergillus species and found homologs of YPS3 (Fig. 4). Interestingly, these homologs entirely lack an intragenic tandem repeat element. It has not yet been determined whether these homologs direct protein production; however, reverse transcription-PCR analysis revealed that transcript is detectable in Aspergillus fumigatus (data not shown). The Aspergillus homologs present the most minimal EGF-like locus, while BAd1 has a large intragenic tandem repeat element. Yps3p falls in between these two extremes, and it will be interesting to determine what effect this variable tandem repeat region has on pathogenesis or other phenotypes.

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