Localization of a Yeast-Phase-Specific Gene Product to the Cell Wall in *Histoplasma capsulatum*

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A yeast-phase-specific gene, *yps-3*, has been identified in the virulent *Histoplasma capsulatum* strain, G217B. Although DNA sequencing of the genomic *yps-3* gene from G217B failed to detect homologies with known proteins, the 5' end of a *yps-3* cDNA contained a consensus signal sequence. A 519-bp fragment of the cDNA containing the translational stop codon was linker modified and inserted into the bacterial expression vector, pATH 1. *Escherichia coli* extracts containing the pATH 1 vector alone expressed a major 34-kDa TrpE polypeptide following induction with indoleacrylic acid, while the pATH 1/*yps-3* construct produced a predominant 54-kDa TrpE/*yps-3* fusion protein. Polyclonal rabbit sera directed against G217B reacted exclusively with the 54-kDa fusion protein in Western blots (immunoblots); serum samples from three patients with acute pulmonary or disseminated histoplasmosis were also positive. To localize the *yps-3* protein within G217B, a monoclonal antibody (MAb 7.1) which recognized the *yps-3* portion of the fusion protein was generated. A 17.4-kDa protein was detected with MAb 7.1 in Western blots prepared from cell wall fractions of G217B; cytoplasmic fractions were unreactive. No *yps-3* antigen was detected in either fraction of the Downs strain, which fails to express the *yps-3* gene. MAb 7.1 also detected a 17.4-kDa antigen in ethanol-precipitated culture supernatants derived from G217B. These findings localize the *yps-3* gene product to the cell wall and culture supernatants, where the protein may influence the phase transition or the maintenance of the yeast state.

We have previously described the cloning and characterization of the yeast phase-specific gene, *yps-3*, from *Histoplasma capsulatum*, a dimorphic fungal pathogen (20). *yps-3* is a nuclear gene whose expression correlates with virulence and morphogenesis when the hyphal form of the organism is removed from ambient temperature and placed at 37°C, when it is converted in vitro and in vivo to the parasitic yeast form (20). The gene is expressed as early as 1 day into the temperature-induced mycelium-to-yeast conversion (20); *yps-3* is expressed at high levels in the virulent G217B strain of *H. capsulatum*, a class 2 strain prevalent in North America, as shown by restriction fragment length polymorphism analysis of ribosomal and mitochondrial DNA (37) and by arbitrarily primed PCR (22). In contrast, the gene is transcriptionally silent in the temperature-sensitive, attenuated Downs strain (10) in both terminal phases and throughout transitions performed at 37 or 34°C (18, 20).

The *yps-3* gene has significance as a diagnostic tool to identify and classify *H. capsulatum* strains in clinical specimens in a typing scheme based on polymorphisms at the nuclear gene locus (19). The gene is apparently *Histoplasma* specific, since the *yps-3* probe fails to hybridize under low-stringency conditions to other medically relevant fungi including *Blastomyces dermatitidis*, *Sepedonium chrysospermum*, *Chrysosporium keratinophilum*, *Saccharomyces cerevisiae*, and various *Candida* species (19, 21). Our laboratory is exploiting knowledge of the nucleotide sequence of *yps-3* (18), and its predicted protein product, in an effort to devise rapid, sensitive tests for the detection of *Histoplasma* species in clinical specimens for diagnosis and as potential monitors for antifungal therapy.

Analysis of the gene or product of the *yps-3* gene has been

unrevealing, as neither the nucleotide sequence nor the predicted amino acid sequence bears homology to sequences currently residing in GenBank (18). As an alternate approach to exploring the function of this yeast phase-specific gene in morphology and virulence in *H. capsulatum*, a 519-bp segment of the *yps-3* cDNA was ligated in frame with the *Escherichia coli* *trpE* gene to generate a fusion peptide. A monoclonal antibody, designated MAb 7.1, specific for the *Histoplasma* segment of the fusion protein was identified and used to localize the *yps-3* gene product to the cell wall in the high-virulence strain G217B. Moreover, several serum samples obtained from patients with disseminated or acute pulmonary histoplasmosis demonstrated reactivity to the predominant 54-kDa *E. coli*-*Histoplasma* fusion protein product of the *trpE*/*yps-3* construct but not to protein induced from cells harboring the pATH 1 control plasmid. These findings demonstrate that *Histoplasma* genes may be cloned and expressed in bacteria and that the protein products are immunogenic. These and other phase-specific reagents, may be useful in the evaluation of the host pathogen response in histoplasmosis.

MATERIALS AND METHODS

Strategy for cloning the pTZ_{yps-3.3} cDNA into a pATH1 bacterial expression vector. The previously described *yps-3* cDNA (18) was isolated from a yeast phase-specific cDNA library representing the virulent strain, G217B, and was constructed in the phagemid vector pTZ (Invitrogen). The pTZ_{yps-3.3} cDNA (18) was digested with *Sma*I within the coding sequence of the gene. The blunt-ended, linearized clone was modified with *Bam*HI linkers (New England Biolabs) and then digested to completion with *Bam*HI and *Hind*III. The material was electrophoresed on a 0.8% agarose gel in 1× Tris-borate-EDTA (TBE) (6), and the modified 519-bp *yps-3* cDNA fragment was isolated by using Gene Clean (BIO101) with the borate buffer modifier. The fragment was ligated (4) into *Bam*HI-*Hind*III-restricted pATH1 (36), a bacterial expression vector which is a derivative of pBR322 (AMP^r gene and origin of replication) containing the *trpE* gene and promoter regions. The ligation mixture was transformed into *E. coli* DH5α (4, 27), and positive recombinants were identified by dot-blot analysis (28) with a nick-translated (30) ³²P-labelled 1.85-kb *Hind*III fragment containing the genomic *yps-3* clone as the probe (20, 35). Positive DNA was then prepared by a miniprep procedure (2, 17) and used to transform *E. coli* C600 (1), a strain with

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a defective heat shock response and limited intracellular proteolysis. Limited dideoxy sequencing of approximately 300 bp of the pATH 1/*yps-3* construct confirmed the insertion of the *yps-3* gene in the correct reading frame (data not shown). For the sequencing analysis, the 620-bp *EcoRI-HindIII* fragment was inserted into M13mp19 and phage DNA was sequenced by the dideoxy chain termination method with Sequenase version 2.0 (U.S. Biochemicals).

Preparation of protein extracts and SDS-polyacrylamide gel electrophoresis.

All C600 cells harboring pATH 1 or derived constructs were maintained on minimal medium containing 5 g of Casamino Acids per liter, 20 mg of tryptophan per liter, and 50 µg of ampicillin per ml. Control C600 cells were maintained on similar plates lacking antibiotic. Stationary-phase cells were diluted 1:10 in minimal medium with Casamino Acids in the absence of tryptophan and grown with shaking for 1 h at 30°C. Expression of the *trpE* gene was induced by the addition of indoleacrylic acid (Sigma Chemical Co., St. Louis, Mo.) to 40 µg/ml of culture and growth of the culture overnight at 30°C with vigorous aeration. Insoluble proteins were prepared as described by Spindler et al. (36). Briefly, 2 ml of overnight culture was pelleted and the cells were resuspended in 100 µl of TEN buffer (50 mM Tris, 0.5 mM EDTA, 0.3 M NaCl [pH 7.5]). The cells were lysed by adding 10 µl of lysozyme (10 mg/ml) for 15 min on ice and then adding 2 µl of 10% Nonidet P-40 for 10 min on ice. Contaminating DNA was removed by incubation for 1 h with DNase I (Sigma) at 37°C. The proteins were pelleted in and washed twice in TEN buffer. Following the final centrifugation, proteins were resuspended in 40 µl of cracking buffer (0.01 M sodium phosphate, 1% β-mercaptoethanol, 1% sodium dodecyl sulfate [SDS], 6 M urea [pH 7.2]) and incubated for 30 min at 37°C. Approximately 2 to 10 µl of this material was loaded on SDS-12.5% polyacrylamide gels prepared by the method of Laemmli (24). Protein markers (Rainbow markers; Amersham) were electrophoresed with extracted samples.

For the isolation of the TrpE/*yps-3* fusion protein for immunization, approximately 100 µl of the insoluble protein was loaded on a ProSieve agarose minigel (FMC Corp.) and electrophoresed in Laemmli buffer (24). The 54-kDa fusion protein was excised relative to prestained markers (Amersham), isolated as recommended by the manufacturer, and concentrated on an Amicon filter. The protein concentrate was lyophilized and stored at -80°C prior to use as the immunogen.

Electrotransfer, preparation of sera, and immunodetection. Following electrophoresis, proteins were transferred for a minimum of 3 h by electroblotting to nitrocellulose (Schleicher & Schuell, Keene, N.H.) with a Transphor apparatus (Hoefer Scientific) at 30 mA in transfer buffer (25 mM Tris-HCl [pH 8.3], 192 mM glycine, 20% [vol/vol] methanol). The blots were stored at 4°C prior to use.

Polyclonal rabbit antisera (38, 39) directed against heat-killed G217B and aliquots of sera from patients with disseminated or pulmonary histoplasmosis were the generous gift of George Kobayashi. Immunoglobulin fractions were obtained from all sera by the addition of saturated ammonium sulfate to 50% followed by precipitation and exhaustive dialysis against phosphate-buffered saline (PBS) (14) (100 mM NaCl, 6 mM Na₂HPO₄, 0.45 mM KH₂PO₄ [pH 7.7]).

The blots were processed for immunodetection by washes in 5% low-fat dry milk (Carnation Instant) in PBS. Binding of rabbit anti-G217B (Y) was detected directly by using a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (heavy- and light-chain specific [Cappel]) followed by development with 4-chloro-1-naphthol and hydrogen peroxide (14). Detection of *yps-3* antibodies in sera obtained from four patients with histoplasmosis by direct methods with peroxidase-conjugated goat anti-human immunoglobulin reagents was weakly successful only for one patient (patient 1 [see below]). To improve sensitivity, primary human immunoglobulin obtained from patient sera (1:500 dilution) was exposed to blots overnight at room temperature; biotinylated goat anti-human immunoglobulin (heavy- and light-chain specific [U.S. Biochemicals]) was subsequently used as a secondary antibody. Streptavidin conjugated to horseradish peroxidase was then applied to amplify the signal, and the blots were processed as described above.

Patients 1 (male) and 3 (female) were both diagnosed in 1971 with disseminated histoplasmosis, and patient 1 had some central nervous system involvement. Both patients were treated with amphotericin B, but with different outcomes: patient 3 responded, but patient 1 died. Patient 2, a male diagnosed with acute pulmonary histoplasmosis in 1981, received no treatment but survived. Patient 4, a female diagnosed in 1969 with disseminated histoplasmosis and unusual vaginal and joint involvement, was treated with amphotericin B and responded. Patient 4 was the index case for infection by the Downs strain of *H. capsulatum* (10).

Immunization, fusion, and MAb screening. Initial intraperitoneal mouse immunizations (15) were done with 20 µg of 54-kD TrpE/*yps-3* fusion protein in complete Freund's adjuvant. Incomplete Freund's adjuvant was used in the four subsequent boosts of 20 µg of fusion protein per boost. A positive test bleed was performed 8 days following the last boost. Fusion of immune splenocytes and preconditioned myeloma P3X63.AG8.653 (15, 34) cells was performed by the gentle addition of 50% polyethylene glycol by the method of Galfre and Milstein (9). Positive growth was observed 10 days postfusion, and screening was performed with nitrocellulose containing either electroblotted 54-kDa TrpE/*yps-3* fusion protein or 34-kDa TrpE protein.

For MAb screening, a *yps-3*-specific enzyme-linked immunosorbent assay ELISA was used (34). Clone supernatants were initially analyzed for reactivity with nitrocellulose filters harboring the 54-kDa TrpE/*yps-3* fusion protein; pos-

itive samples were then screened for the absence of reactivity with the 34-kDa TrpE protein on nitrocellulose filters. MAb 7, an immunoglobulin G1 producer, reacted specifically with the fusion peptide but failed to bind to nitrocellulose filters electroblotted exclusively with TrpE protein. For the screenings, the filters were blocked for 30 min in 5% nonfat dry milk in PBS, rinsed twice in PBS, and incubated by rotation at 23°C overnight with culture supernatants. The filters were blocked twice in 5% dry milk for 5 min, rinsed in PBS, and then incubated for 1 h with peroxidase-conjugated goat anti-mouse antibody (heavy- and light-chain specific; U.S. Biochemicals) followed by biotin swine anti-goat antibody (CalTag) to amplify the signal. The final reagent, peroxidase-conjugated streptavidin (Zymed), was applied for 1 h. The filters were developed in 4-chloro-1-naphthol and peroxidase substrate (14). Single-cell cloning was achieved by limiting-dilution analysis followed by expansion (15, 34) to obtain MAb 7.1.

Generation and characterization of MAb 7.1. To produce a MAb suitable for subcellular localization of the *yps-3* protein, the 54-kDa TrpE/*yps-3* protein was exploited as an immunogen in BALB/c mice. The fusion partner for immune splenocytes was the murine myeloma P3X63Ag8.653 (15, 34). Initial screening was based on growth positivity in individual fusion wells followed by positive ELISA reactivity with a solid-phase recombinant TrpE/*yps-3* protein expressed in *E. coli*. To exclude the possibility that positive clones were recognizing the TrpE portion of the immunogen, clones were screened for negative ELISA reactivity with solid-phase nitrocellulose filters harboring the 34-kDa TrpE protein expressed by C600 cells harboring only the pATH 1 vector (30).

On analysis, 72% of the fusion wells (378 of 526) were growth positive. Of the growth-positive wells, 9.5% exhibited ELISA reactivity with the TrpE/*yps-3* fusion protein but 34 of 36 clones were also positive in the 34-kDa TrpE ELISA. Two clones, representing 5.6% of the antibody-positive cultures, were reactive against the *yps-3* portion of the recombinant protein by this ELISA screening protocol. One clone was determined to be an IgM producer (data not shown) and was not characterized further. MAb 7, an immunoglobulin G1 producer, was cloned by limiting dilution to generate MAb 7.1 and expanded for additional analysis.

Preparation of cell wall, cytosol, and supernatant fractions from *H. capsulatum* strains in culture. Strain G217B (ATCC 26032) or Downs (ATCC 38904) cells were grown at 37°C with gentle shaking for 48 h in liquid GYE (2% glucose, 1% yeast extract) supplemented with cystine (8.4 µg/liter). The cells were pelleted and resuspended in PBS (pH 7.2)-0.01% thimersol for 1 h, essentially as described by Gomez et al. (11). The cells were washed in breaking buffer (1 mM phenylmethylsulfonyl fluoride, 5 µM leupeptin, 0.15 mM pepstatin, and 50 mM EDTA in PBS) and then resuspended in 2 volumes of breaking buffer. They were broken in a bead beater (Biospec Products) on ice for 7 min of alternating 30-s cycles. Homogenized cells were centrifuged for 20 min at 5,000 rpm (SS34 rotor; Sorvall), and the supernatant was further centrifuged for 20 min at 25,000 rpm (SW28 rotor; Beckman). The pooled supernatant fractions were dialyzed against PBS as the cytosolic fraction. The pooled particulate fractions were washed three times in PBS, solubilized in 2 volumes of boiling buffer (125 mM Tris, 6 M urea, 1% Tween 20, 1.7% β-mercaptoethanol) for 2 min at 100°C, and then rocked overnight at 4°C. The final cell wall fraction was obtained following centrifugation for 20 min at 25,000 rpm (SW28 rotor), and dialysis against PBS.

Supernatant fractions were obtained from strain G217B grown at 37°C for 3 days with shaking in liquid GYE medium supplemented with cystine. Cells were pelleted at 5,000 rpm (SS34 rotor), and the supernatant was precipitated by the addition of 2 volumes of absolute ethanol (30 min at 23°C) followed by centrifugation at 10,000 rpm (SS34 rotor) for 30 min. The particulate fraction was resuspended in PBS.

RESULTS

Polyclonal antibody detection of the TrpE/*yps-3* fusion protein. A 519-bp *SmaI-HindIII* fragment of the pTZ_{yps-3.3} cDNA, containing the open reading frame and including the *yps-3* translational stop codon and a 3' nontranslated region, was inserted into the pATH 1 vector, a member of a group of bacterial expression plasmids (pATH series) which permit insertion and expression of a heterologous gene at the *BamHI* site to generate fusion proteins encoded by any of three reading frames (Fig. 1). Insertion into the *BamHI* site produces a deletion of the 3'-most end of the *trpE* gene (and thus the loss of the bacterial carboxy terminus), resulting in a final fusion protein that contains the entire N-terminal region of the TrpE protein fused in frame to the *yps-3* protein supplied by the fungal open reading frame. Although sequencing analysis of the *yps-3* cDNA (18) suggested that the pATH 1 vector was appropriate for in-frame fusion protein production, similar *BamHI* linker modified constructs of the *yps-3* cDNA were inserted into pATH 2 and pATH 3 vectors (36).

In the stained polyacrylamide gel (Fig. 2A), the pATH 1

FIG. 1. Cloning strategy for the pATH 1/*yps*-3 construct. The pATH vector is a derivative of pBR322 (Amp^r and replication origin) containing the *trpE* gene and promoter region. The *E. coli trpE* gene encodes a 34-kDa protein. A polylinker region has been inserted near the 3' end of the *trpE* gene; the vector series is designed so that insertion of a heterologous gene at the *Bam*HI site can generate fusion proteins in any of three reading frames. Limited dideoxy DNA sequencing confirmed insertion of the *yps*-3 gene in the correct reading frame in pATH 1. Additional fusion constructs were made with using linker-modified *yps*-3 sequences in pATH 2 and 3. Only short fusion proteins were expressed upon induction in these vectors, and the expressed proteins did not react with polyclonal rabbit antisera directed against G217B (data not shown).

vector containing the intact *E. coli trpE* gene expresses a predominant 34-kDa protein and a minor 31.5-kDa band under induced conditions with indoleacrylic acid. In contrast, the major protein expressed in the pATH 1/*yps*-3 construct was 54 kDa, with a broadly stained set of bands in the range of 36 to 44 kDa. Western blot analysis of the pATH1/*yps*-3 construct under indoleacrylic acid induction of the *trpE* operon demonstrated a 54-kDa fusion protein that was detected with a polyclonal rabbit antiserum directed against G217B (Fig. 2B). The immune rabbit immunoglobulin failed to react with proteins produced under induced or noninduced conditions in C600 cells harboring pATH 1 vectors alone (Fig. 2B) or from the pATH 2/*yps*-3 or pATH 3/*yps*-3 constructs (data not shown). These findings suggested that the *yps*-3 cDNA had been inserted correctly in frame with the *trpE* gene in the pATH 1 vector and that the native *yps*-3 protein from G217B was immunogenic.

Material in the 36- to 44-kDa range observed in the pATH 1/*yps*-3 construct under induction was also immunoreactive with the rabbit immunoglobulin under overnight induction conditions, as seen in Fig. 2B. However, when induction was optimized for a 12-h period, the 34- to 44-kDa proteins were not apparent in stained gels or Western blots (data not shown). It was likely that some protein degradation had occurred during the longer induction, and the 12-h induction period was used in subsequent experiments.

Amino-terminal sequence analysis of the predicted *yps*-3 protein. The partial *yps*-3 cDNA inserted into the expression vector lacks the first 24 N-terminal amino acids predicted on

FIG. 2. Western blot analysis of the TrpE/*yps*-3 fusion protein and controls with polyclonal rabbit immunoglobulin fractionated sera prepared against G217B. (A) SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide) of control or fusion proteins under induced (+) or uninduced (-) conditions. Protein extracts were prepared from C600 cells harboring the pATH 1 plasmid alone or from cells transformed with the pATH 1/*yps*-3 construct. β -Indoleacrylic acid was used to induce the expression of the *trpE* gene in both clones (+ lanes); the profile of proteins obtained from cells in the absence of induction (- lanes) is also seen in the Coomassie brilliant blue-stained gel. (B) Western blot analysis of the transferred gel. Nitrocellulose blots were obtained from Western transfer of unstained proteins electrophoresed in panel A. The blot was incubated with the immunoglobulin fraction obtained from a polyclonal rabbit antiserum directed against G217B. The blot was then processed and developed as indicated in Materials and Methods with horseradish peroxidase as the indicator enzyme. The polyclonal antibodies recognized the 54-kDa TrpE/*yps*-3 fusion protein under induced conditions but failed to react with extracts prepared from C600 cells harboring the pATH 1 plasmid. Some degraded forms of the fusion protein (at about 39 kDa) may also be reacting.

the basis of S1 nuclease analysis (18) and primer extension studies (data not shown). Previous sequence analysis has failed to find significant homology between the *yps*-3 gene product and other proteins at the nucleotide or protein level (18, 19). To determine the role that these N-terminal residues might play in the authentic *yps*-3 protein, the putative *yps*-3 protein was subjected to hydrophobicity analysis by the algorithm of Kyte and Doolittle (23). The first 32 N-terminal residues of the *yps*-3 protein were found to contain a consensus endoplasmic reticulum (ER) import sequence (Fig. 3). A stretch of 13 hydrophobic amino acids in the *yps*-3 protein (Thr-Leu-Leu-Leu-Leu-Ala-Ser-Ser-Ser-Leu-Leu-Val-Ala-Ala-Arg-Pro-Gly-Glu-Tyr-Pro-Thr-Asp-Lys-Asp, compared with the ER signal sequence of Met-Met-Ser-Phe-Val-Ser-Leu-Leu-Leu-Val-Gly-Ile-Leu-Phe-Trp-Ala-Thr-Glu-Thr-Glu-Ala-Glu-Gln-Leu-Thr-Lys-Cys-Glu-Val-Phe-Gln, where the underlined amino acids represent hydrophobic regions (41). Both the *yps*-3 and ER import sequences contain three net negative charges and one net positive charge. The algorithm revealed a second hydrophobic region of the *yps*-3 protein

FIG. 3. Hydrophobicity plot of the predicted yps-3 protein. The output is a list of hydropathy values for each subsequence of 7 amino acids and a histogram-like plot of these values along the sequence, based on the algorithm of Kyte and Doolittle (23). Regions with hydropathy values equal to or in excess of +1.6 are hydrophobic. One major hydrophobic leader region, near the amino terminal of the predicted protein, is homologous to the ER signal sequence. No signal peptide sequence specific for retention in the ER lumen is observed. A second, small hydrophobic region near the COOH terminus was observed.

localized in the C terminus of the polypeptide (Phe-Pro-Ser-Ile-Leu-Asn-Thr-Leu-Leu-Ala-Leu-Phe-Val-Cys-Asn-Gly). This second hydrophobic region (region 2) of the yps-3 protein was insufficient to span the membrane as a hydrophobic α helix (Fig. 3) but had features homologous to glycosylphosphatidylinositol (GPI) linkage signals for cell wall proteins of eukaryotes (3), including *S. cerevisiae* (25, 26, 32, 43). The signal that specifies this type of covalent modification is contained within the hydrophobic carboxyl-terminal sequence and adjacent residues, which include Asp and a nonbulky aliphatic amino acid (3). Because of this lipid anchor, the protein remains membrane bound with all of its amino acids initially on the luminal side of the ER and eventually on the cell exterior. These observations suggested that the yps-3 protein might be localized in the cell wall of *H. capsulatum*.

Localization of the yps-3 protein to the cell wall in *H. capsulatum* G217B. The yeast phases of G217B and the Downs strains were fractionated under denaturing conditions in urea by the method of Gomez et al. (11) with modifications. Cell wall and cytosolic fractions were obtained from the yeast phase of each isolate; a membrane fraction obtained by a repetitive freeze-thaw regimen oscillating between -80 and 4°C was the generous gift of George Deepe. As shown in Fig. 4, MA b 7.1 reacted with a 17.4-kDa polypeptide found exclusively in the cell wall fraction of G217B. The detected size is somewhat smaller at 17.4 kDa than the molecular mass predicted on the basis of amino acid sequence (approximately 19 kDa). Covalent attachment of the complete precursor yps-3 protein to the fungal membrane via a glycosylphosphatidylinositol anchor would require loss of N-terminal amino acids (prior to the signal peptidase cleavage site) and a C-terminal truncation of the polypeptide (with concomitant loss of approximately 24 residues, including the 16-amino-acid hydrophobic sequence and adjacent residues), presumably resulting in the smaller observed size. No immunoreactive proteins were found in the freeze-thaw sample or the cytoplasmic fraction of G217B or in the cell wall and cytosolic samples obtained from the Downs strain. These findings are consistent with the observation that the yps-3 gene is transcriptionally silent in both the hyphal and yeast phases of the low-virulence Downs strain. Moreover,

these studies suggest that a strongly denaturing regimen (6 M urea) with a nonionic detergent (Tween 20) is required for liberating the yps-3 protein from the cell wall of the intact yeast, as might be predicted by a GPI-anchored protein.

Culture supernatants from G217B contain the yps-3 protein. The modest size of the 17.4-kDa yps-3 protein detected by Western blotting in the cell wall of G217B (Fig. 4) was reminiscent of many growth factors and cytokines (7, 15). To determine if the yps-3 polypeptide might be secreted into the yeast culture medium or cleaved at the lipid anchor to release the extracellular protein, GYE used to support the growth of G217B strain yeasts for 3 days was harvested first by centrifugation and then filtration to remove any residual organisms.

FIG. 4. Fractionation of yeast cells from the *H. capsulatum* G217B and Downs and Western blot analysis. The yeast phase of G217B and Downs was fractionated under denaturing conditions in urea by the method of Gomez et al. (11) into cell wall (CW) and cytosolic (Cyto) fractions. A cell wall sample from G217B was obtained by a freeze-thaw regimen (FT) and was the generous gift of George Deepe. Approximately 10 μg of each fraction was electrophoresed on an SDS-12.5% polyacrylamide gel; 75 μg of the corresponding Downs fraction was electrophoresed in parallel. The gel was electroblotted and processed as described in the text, with MA b 7.1 as the primary antibody.

FIG. 5. Western blot analysis of proteins extracted from culture supernatants obtained from G217B yeasts. Proteins from culture media used to support the growth of 3-day-old G217B yeast cultures were recovered by ethanol precipitation, and a 20- μ g sample was electrophoresed with cytoplasm and freeze-thaw samples from G217B. A dominant band with an approximate molecular mass of 17.4 kDa is observed, but a faint signal is detected at 19 kDa, the size of the yps-3 protein predicted on the basis of amino acid composition.

Proteins were recovered by ethanol precipitation, and a 20- μ g sample was electrophoresed with cytoplasm and freeze-thaw samples from G217B (Fig. 5). Western blot analysis with MAb 7.1 revealed a major 17.4-kDa immunoreactive protein in the proteins obtained from the yeast culture supernatant (Fig. 5).

Sera from patients with acute or disseminated histoplasmosis react with the induced *E. coli* (TrpE)-*Histoplasma* (yps-3) fusion protein. Since the native yps-3 protein could be found in culture supernatants and might be emitted from free yeast cells in the infected host, sera from four patients were evaluated for yps-3 protein antibody by an enzyme-linked detection assay.

The proteins were prepared from C600 cells harboring the pATH 1 and pATH 1/yps-3 constructs, electrophoresed, and either stained (Fig. 6A) or transferred by electroblotting (Fig. 6B) for Western blot analysis with partially purified patient sera enriched for the gamma globulin fraction. Three of the four specimens from patients 1, 2, and 3 reacted exclusively with the induced 54-kDa TrpE/yps-3 fusion protein but not with the 34-kDa protein expressed from C600 cells with the pATH 1 construct. In contrast, the serum sample from patient 4, infected with the Downs strain of *H. capsulatum* (10), failed to react with any of the proteins in this Western blot analysis, even when a detection scheme involving biotinylated goat anti-human antibody and horseradish peroxidase-conjugated streptavidin was used.

DISCUSSION

In this study, a complete open reading frame including the translation termination signal and a significant 3' nontranslated region of the cDNA encoding a yeast phase-specific gene, yps-3, isolated from the virulent strain G217B of *H. capsulatum* was cloned in frame in the pATH vector expression series (36). Only the pATH 1/yps-3 construct expressed a 54-kDa *E. coli*-*Histoplasma* fusion protein consistent with the nucleotide sequence of the *trpE* and *yps-3* genes to result in a product reactive with polyclonal G217B anti-yeast rabbit antisera. Induction of a slightly truncated, 34-kDa TrpE protein was observed when control pATH 1 plasmid was transformed into C600 cells, but polyclonal rabbit antisera (Fig. 2B) and sera obtained from patients with histoplasmosis (Fig. 6B) failed to react in Western blots with this *E. coli* product. The expressed

FIG. 6. Western blot analysis of TrpE/yps-3 fusion proteins with antisera derived from patients with histoplasmosis. (A) SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide) of TrpE/yps-3 fusion proteins. Proteins were prepared from C600 cells harboring the pATH 1 vector or transformed with pATH 1/yps-3 constructs. Extracts were also prepared from C600 cells as control as described in Materials and Methods. Proteins were stained with Coomassie brilliant blue. (B) Western blot analysis of patient sera. Western blots were processed with the immunoglobulin fractions derived from four patient serum samples. Secondary antibody binding was accomplished with biotinylated goat anti-human antibody followed by reaction with horseradish peroxidase-conjugated streptavidin. Three of the four patient specimens (Pt. 1, 2, and 3) reacted with the 54-kDa TrpE/yps-3 fusion protein under these conditions. The fourth patient (Pt. 4) was the index case for infection by *H. capsulatum* Downs.

54-kDa fusion protein induced in C600 cells with the pATH 1/yps-3 construct was purified and used to develop a MAb, MAb 7.1, which reacted with the *Histoplasma* segment of the fusion protein but lacked reactivity with the bacterial leader. MAb 7.1, directed against the open reading frame of the native yps-3 mRNA, was used to localize the 17.4-kDa yps-3 gene product to the cell wall in G217B, consistent with an observed ER import sequence and a second hydrophobic region reminiscent of GPI-linked proteins in the cell wall proteins of *S. cerevisiae* (25, 26, 32, 43). As anticipated, the MAb was unable to detect the protein product in cell wall fractions prepared from the attenuated Downs strain of *H. capsulatum* (10), which is devoid of yps-3 transcription in the yeast and hyphal phases (18, 20).

Several features of the predicted amino acid sequence of the yps-3 polypeptide (18) suggest that it is cotranslationally inserted into the rough ER and posttranslationally modified by transfer to glycolipid. The predicted amino terminus of the protein has a consensus ER import sequence (41) and a canonical signal peptidase cleavage site (40). Moreover, all GPI-linked proteins are synthesized with cleavable sequences at their carboxy termini, which have been deduced by analyzing the sequences flanking the sites of anchor addition (3). The yps-3 protein has a moderately hydrophobic C-terminal sequence of 16 amino acids (Fig. 3), lacks a potential cytoplasmic domain (Fig. 3), and has nearby Ser residues which conform to identified GPI attachment sites (3). The protein also has multiple sites for O-linked glycosylation via serine or threonine residues in the central region (18) but lacks consensus sites for N-linked glycosylation via asparagine residues. These observations and the biochemical localization findings reported here suggest that the yps-3 protein is a cell wall protein in *H. capsulatum*, covalently attached to the outer face of the cell membrane. Immunofluorescence studies with intact G217B cells

and the MAb reagent described in this study have been unsuccessful at verifying this prediction: our preliminary observations showed a high autofluorescent background on the yeast cell surface, also, extensive glycosylation in the glycan layer, potentially at the yps-3 protein itself, may have obscured the epitope (data not shown). Although the physiologic functions of most GPI-anchored proteins are unknown, many have enzymatic or cell adhesion properties. In the yeast *S. cerevisiae*, the cell surface agglutinins involved in the mating response are both GPI linked. α -Agglutinin contains a high proportion of O-linked carbohydrate and has two subunits: the AGA1 gene product (32) is involved in cell surface attachment, while the AGA2 product contributes the binding subunit which interacts with α -agglutinin. In contrast, the α -agglutinin expressed on the opposite mating type is a single GPI-linked glycoprotein which is the product of the AGA1 gene (25) and is a member of the immunoglobulin family of cell adhesion proteins (43). Several GPI-linked precursors of α -agglutinin, including a 140-kDa ER precursor (43), a high-molecular-weight glucosylated plasma membrane form, and a soluble form (26) which is apparently a periplasmic intermediate that is cross-linked to the cell wall β -glucan and lacks the GPI anchor (26), have been identified. The 17.4-kDa soluble yps-3 protein found in yeast culture supernatants (Fig. 5) may thus represent polypeptide liberated from the lipid anchor; the faintly immunoreactive band at 19 kDa in this blot (Fig. 5) may be unmodified and unlinked protein committed to the secretory pathway.

Early events in histoplasmosis result in a pneumonia in which phagocytic cells, including neutrophils and macrophages, play a key role. Yeasts localize in the phagolysosomes of infected macrophages, where they continue to replicate in spite of reactive oxygen and nitrogen metabolites and eventually disrupt cellular integrity. Although macrophages are certainly the principal effector cells participating in host defense against *H. capsulatum*, several lines of evidence, including studies based on infection in nude mice lacking mature T-cell populations (42) and adoptive transfer experiments (5), have implicated CD4⁺ T cells in the host defense repertoire. The role of the CD4⁺ T-cell population is unclear, since these cells can mediate both cellular and humoral effects. For example, gamma interferon (45) and macrophage and granulocyte-macrophage colony-stimulating factors (29) act to limit the intracellular growth of *Histoplasma* yeast cells in murine and human macrophage models, apparently at the cellular level. A survey of the predicted amino acid sequence of the yps-3 antigen (18) by sequence pattern analysis predicts several IA^d- and IE^d-binding regions (33). IE^d-binding motifs representing the "basic-basic-noncharge-basic" consensus (33) are predicted to occur in the yps-3 protein near the N terminal region (amino acids 34 to 41) and in the internal segment of the protein (residues 67 to 72 and 135 to 140). Potential T-cell receptor interaction sites for yps-3 peptide (18) in the context of IA^d (33) are also observed in the central region (residues 60 to 65), as well as in the C-terminal region (residues 166 to 171) which is likely to be removed during membrane lipid anchoring. It is thus likely that the cell wall yps-3 antigen may contribute to the host cell-mediated response in histoplasmosis. However, it is unclear if the expressed yps-3 protein will prove protective, although peripheral blood mononuclear cell cultures from individuals cured of histoplasmosis proliferate in response to the 54-kDa TrpE/yps-3 fusion protein but not to the bacterial TrpE protein alone (4a). It will be necessary to purify the native protein with the reagents generated in this study to further explore the role that the yps-3 antigen may play in cellular immunity to *H. capsulatum*.

In contrast, the H and M glycoprotein antigens (13, 44),

initially detected by an immunodiffusion screen for abundant phase-specific antigens (12), clearly stimulate a humoral response in the host, with circulating anti-H antigen antibodies serving as a diagnostic signal for active histoplasmosis. Interestingly, the observations reported here indicate that the infected human host can mount a humoral response against the yps-3 cell wall antigen, which can be assayed in the serum of patients with pulmonary or disseminated histoplasmosis. However, the general utility of the Western blot assay reported in this study may be restricted by the infecting *Histoplasma* strain. This study suggests that the positioning of the yps-3 protein in the cell wall of *H. capsulatum* correlates with temperature sensitivity and virulence in nonisogenic strains, in which it may effect morphotype conversion and influence the host-pathogen interaction.

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