Glycosylation and Immunoreactivity of the *Histoplasma capsulatum* Cfp4 Yeast-Phase Exoantigen

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The yeast phase of *Histoplasma capsulatum* is the virulent form of this thermally dimorphic fungal pathogen. Among the secreted proteome of *Histoplasma*, culture filtrate protein 4 (Cfp4) is a heavily glycosylated factor produced abundantly and specifically by *Histoplasma* yeast cells, suggesting its role in pathogenesis. We have generated three monoclonal antibodies as tools for characterization and detection of Cfp4 and determined the epitope each recognizes. Through site-directed mutagenesis of Cfp4, we identified three asparagines that function as the principal sites of N-linked glycan modification. To test the function of Cfp4 in *Histoplasma* pathogenesis, we generated Cfp4-deficient strains by insertional mutagenesis and by RNA interference. Cfp4-deficient strains are not attenuated in virulence in human macrophages or during lung infection in a murine model of histoplasmosis. Coinfection of differentially marked Cfp4-producing and Cfp4-deficient strains demonstrates that production of Cfp4 does not confer a fitness advantage to *Histoplasma* yeasts during murine lung infection. Despite no apparent role in acute virulence in mice, secretion of the Cfp4 glycoprotein by yeast cells is consistent across clinical and laboratory isolates of the North American type 1 and type 2 phylogenetic groups as well as a strain from Panama. In addition, human immune sera recognize the *Histoplasma* Cfp4 protein, confirming Cfp4 production during infection of human hosts. These results suggest the potential utility of Cfp4 as a diagnostic exoantigen for histoplasmosis.

*Histoplasma capsulatum* is a member of the thermally dimorphic group of fungal pathogens that infect humans and other mammals, causing respiratory and systemic disease in these hosts (1–3). At ambient temperatures in the soil, *Histoplasma* grows as a saprobiac conidium-producing mold. Disturbance of the mold form aerosolizes conidia, the inhalation of which initiates respiratory infection. Exposure to 37°C in the mammalian lung triggers a morphological and lifestyle change that results in the differentiation of conidia into yeast cells which parasitize host phagocytes. This conversion to the yeast form and expression of the yeast-phase regulon of genes are essential for the virulence of *Histoplasma* (4–6).

Unlike opportunistic fungi, *Histoplasma* survives the innate immune response largely by production of virulence factors that subvert or inactivate innate defenses (7). *Histoplasma* cells are efficiently taken up by phagocytes, chiefly alveolar macrophages which patrol the alveolar spaces. By expression of an α-linked glucan cell wall polysaccharide, *Histoplasma* yeasts conceal immunostimulatory cell wall β-glucans from detection by phagocytes (8). In addition, *Histoplasma* yeasts express an extracellular oxidative stress response system consisting of the extracellular superoxide dismutase, Sod3, and the extracellular catalase, CatB (9, 10). These secreted and cell surface-localized factors protect *Histoplasma* yeasts from the antimicrobial phagocyte-produced reactive oxygen during uptake by host phagocytes. Within phagocytes, *Histoplasma* yeasts grow and replicate, ultimately leading to lysis of the phagocyte and spread of the infection. A few factors facilitating the intramacrophage lifestyle of *Histoplasma* are beginning to be defined (11) and include Cbp1, a secreted factor of unknown function (12), production of siderophores and iron reductase systems that enable iron acquisition within the phagosome (13–15), de novo synthesis of essential vitamin cofactors (16), and thermotolerance (17).

Secretion is a hallmark of most *Histoplasma* virulence factors identified to date, positioning these factors to directly interact with host cells or molecules (18). As a foundation to better understand the secreted factors that contribute to *Histoplasma*’s interaction with its host, we recently identified a core set of extracellular proteins produced by *Histoplasma* yeast cells (19). Five of the proteins secreted by yeasts lacked significant homology to other identified proteins from other organisms. These were designated culture filtrate proteins (Cfp), and the genes encoding three (Cfp1, Cfp4, and Cfp8) showed higher expression by pathogenic yeasts than by mycelia (19). At the protein level, Cfp4 was one of the most abundant extracellular proteins, second to the Cbp1 secreted factor.

In this study, we further characterize the Cfp4 protein and investigate its contribution to *Histoplasma* pathogenesis. We show that Cfp4 is heavily glycosylated and, through site-directed mutagenesis, identify which amino acids are attachment sites of N-linked glycan. Despite its abundant production, loss of Cfp4 does not reduce the virulence of two distinct strains of *Histoplasma* during acute respiratory infection, nor does Cfp4 provide a competitive advantage in coinfection experiments. Cfp4 is secreted by all strains tested from three different phylogenetic groups of *Histoplasma*. Cfp4 reacts with immune sera from histoplasmosis pa-
tients, providing evidence of production in vivo and suggesting that Cfp4 has potential as a diagnostic exoantigen.

**MATERIALS AND METHODS**

**Culture of Histoplasma yeasts.** *Histoplasma capsulatum* strains (Table 1) included the laboratory strains G186A (ATCC 26027) and G217B (ATCC 26032), clinical isolates obtained from the Ohio State University Clinical Microbiology Lab, and mutants derived from the G186A and G217B backgrounds. *Histoplasma* cells were maintained as yeasts by growth at 37°C in *Histoplasma*-macrophage medium (HMM) (20). For the growth of uracil auxotroph strains, medium was supplemented with uracil (100 μg/ml). Liquid cultures were aerated by shaking (200 rpm) and grown until late exponential/early stationary phase. Growth rate and stage were determined by measuring liquid culture turbidity at 595 nm after addition of culture to 1 M NaOH. For experiments requiring defined yeast numbers, single clones were obtained. The insertion site was determined by sequencing of the PCR product. The T-DNA insertion mutation in strain OSU6 was complemented by transformation of OSU6 with a linearized plasmid containing the Cfp4 cDNA by PCR using primers ACGGCCGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTGCT (amplifying the PH2B-CFP4 gene (amplified by PCR from G186A genomic DNA with primers AGGCCGCCATGAAACACTGTTGTTGCGCTCAATCGTGGTTCAGAACTGCTC and TCAATAATTGTATCCGTTT
control green fluorescent protein (GFP)-RNAi and the CFP4-RNAi plasmids were linearized by digestion with PacI and transformed by electro-
poration (25) into the WU15 uracil auxotroph strain (23). Ura+ transform-
ants were selected and screened for silencing of Cfp4 production by silver staining of culture filtrate proteins.

**Determination of Cfp4 production and glycosylation.** The produc-
tion of Cfp4 by *Histoplasma* yeasts was assessed by silver staining of polyc-
arylactopyranoside (IPTG).

**Supernatants** were concentrated using an Amicon ultrafiltration unit with a 10-kDa-molecular-mass-cutoff polyethersulfone (PES) membrane (Millipore). The protein concentrations of culture filtrates were determined using a DC protein assay with an ovalbumin protein standard (Bio-Rad). A total of 0.5 μg of untreated or PNGase F-treated glycososy-
lated culture filtrate proteins was separated by 10% SDS-PAGE, trans-
ferred to nitrocellulose, and probed with histoplasmosis patient immune serum at 1:500. The histoplasmosis sera were obtained from a 2012 out-
break investigation in Nebraska by the CDC (28). Characteristics of in-
fected individuals are described in reference 29. Sera were previously con-
ferred positive by histoplasmin complement fixation and *Histoplasma*
poly saccharide antibody testing. Being from an outbreak, the cases can be classified as probable acute or subacute pulmonary histoplasmosis. Im-
munoblotting was also performed with pooled high-titer blastomycesis immu-
ne sera prepared from 5 individuals with high reactivity in a *Blastomy-
ces* immunoassay (30). Control healthy human serum was from 5 residual sera from the Indiana Blood Bank chosen because Indiana is an area where *Histoplasma* is endemic. Protein recognition was visualized using biotin-
ylated anti-human secondary antibody (0.15 μg/ml) and streptavidin-
horseradish peroxidase (0.2 μg/ml) with Opti 4CN substrate color detect-
ion (Bio-Rad).

**Macrophage infections.** To determine the role of Cfp4 in macrophage
killing, we utilized the previously described P338D1-lacZ macrophage
killing assay in which macrophage-expressed LacZ is used as an indicator
of surviving macrophages (17). Macrophages were maintained in Ham’s
F-12 with 10% heat-inactivated fetal bovine serum (FBS). Macrophages
were seeded at 2.5 × 10⁴ macrophages per well of a 96-well plate and
incubated at 37°C with 5% CO₂-95% air for the duration of the assay.
Yeasts of strains OSU45, OSU84, OSU18, OSU37, and OSU87 were grown
to late exponential growth phase in HMM and counted on a hemacyt-
ometr. Yeasts were diluted and suspended in HMM-M (HMM buffered to
pH 7.2 with 25 mM bicarbonate) with 10% FBS, and suspensions consist-
ing of 2.5 × 10⁴ yeasts were used to replace the medium on macrophages.
Macrophage monolayers were examined visually daily and compared
to wells containing uninfected macrophages. After 7 days, the number of
surviving macrophages was determined by measuring the total β-gal-
osidase activity in each well (17). Briefly, culture media were removed,
and surviving macrophages were lysed by the addition of phosphate-
buffered saline (PBS) containing 0.5% Triton X-100, 2 mM MgCl₂, and 2
mg/ml o-nitrophenyl-β-D-galactopyranoside (ONPG). Substrate conver-
sion was allowed to proceed for 30 min at room temperature before color
change was read at 420 nm with correction at 595 nm.

**Isolation and infection of human monocyte-derived macrophages.** Peripheral blood monocytes were collected from healthy human volun-
teers, and monocytes were differentiated into macrophages as described
previously (31). Human cells were obtained from healthy volunteers after
obtaining written informed consent and Health Insurance Portability and
Accountability Act (HIPAA) authorization. Human subject research was
approved by the Biomedical Sciences Institutional Review Board at Ohio
State University (protocol 2008H0242). Blood was collected by venipunc-
ture into heparinized tubes. For monocyte collection, 20 ml of heparin-
ized blood was mixed with 15 ml of saline. A total of 14 ml of Ficoll-Paque
PLUS was underlayed to the blood suspension, and cells were separated by
centrifugation (400 g for 40 min at 18°C). The upper plasma layer was
removed, the Ficoll and “buffy coats” were pooled, and the volumes were
brought to 50 ml with saline. Monocytes were collected by centrifugation
(200 × g for 15 min at 4°C) and transferred to Teflon wells in RPMI 1640
medium containing 20% autologous serum at a concentration of 2 × 10⁶
cells/ml. Monocytes were incubated for 5 days at 37°C with 5% CO₂-95% air
for differentiation into macrophages. Cell suspensions were collected by
centrifugation (200 × g for 15 min at 4°C), seeded at 8 × 10⁵ total cells
per well of a 96-well plate in RPMI 1640 medium with 10% autologous
serum, and incubated for 2 at 37°C before removal of nonadherent cells.
For experiments involving activated macrophages, macrophages were
incubated with gamma interferon (IFN-γ) (1,000 U/ml; BioLegend) for 48 h.

For infection of monocyte-derived macrophages, *Histoplasma* yeasts
were grown to early exponential growth phase in HMM and enumerated
by a hemacytometer. Yeasts were added to the wells containing monocyte-
derived macrophages at 2 × 10⁴ yeasts/well, and infections were allowed
RESULTS

Generation of Cfp4-depleted Histoplasma strains. To probe the functional role of Cfp4 in the biology of Histoplasma, we created strains in which synthesis of the CFP4 gene product was prevented by insertional mutation or Cfp4 production was depleted by RNA interference. Cfp4 was originally identified from a proteomics analysis of the secreted proteome of the Panama lineage strain, G186A (19). Transcript sequencing confirmed the exon structure of the CFP4 gene (Fig. 1A). The gene encodes a 213-amino-acid protein of which notable features include a secretion signal at the N terminus and a proline/threonine-rich region (Fig. 1B). In addition, potential N-linked glycosylation sites were identified by bioinformatics analysis (Fig. 1B), and the verification of N-linked glycan modification of Cfp4 is detailed below. The Cfp4 protein is predicted to be 23 kDa in size, although the protein migrates as a broad 31- to 34-kDa band in one-dimensional acrylamide gels after enzymatic removal of N-linked glycosylation. To enable functional tests, we isolated mutants that were unable to produce the Cfp4 protein. We screened random T-DNA insertions to identify a mutant harboring a T-DNA insertion at the CFP4 locus (strain OSU6). The T-DNA insertion in OSU6 is located 209 bp upstream of the initiation codon (Fig. 1A). The insertion disrupts the CFP4 promoter, preventing expression of CFP4 as evidenced by lack of Cfp4 protein in deglycosylated culture filtrates derived from OSU6 yeasts (Fig. 1C). As an independent means of preventing Cfp4 function, we also created Cfp4-depleted lines through RNA interference (RNAi). Transduction of yeasts with the CFP4-
RNAi plasmid, but not with an RNAi plasmid targeting gfp, prevented Cfp4 accumulation in yeast culture filtrates (Fig. 1C). In similar fashion, RNAi was used to suppress Cfp4 production by yeasts of the North American type 2 (NAm2) phylogenetic group (Fig. 1C). Examination of the proteins in the culture filtrate produced by these CFP4-RNAi yeasts showed loss of Cfp4 protein (Fig. 1C).

**Characterization of Cfp4 N-linked glycosylation.** We generated custom monoclonal antibodies to Cfp4 as a tool to track production and posttranslational modification of the Cfp4 protein. Mice were immunized with a chimeric protein containing seven selected Cfp4 epitopes (Fig. 2A). Following hybridoma establishment, the hybridoma culture filtrates were screened for recognition of *Histoplasma* Cfp4. Three hybridoma lines producing antibodies that detect *Histoplasma* Cfp4 were obtained: 2D20, 2F9, and 3G14. The antibodies specifically recognize the *Histoplasma* Cfp4 protein since immunoblots of wild-type *Histoplasma* yeast culture filtrate proteins react with a 33-kDa band but fail to detect anything in culture filtrates derived from mutant yeasts unable to produce Cfp4 (Fig. 2B). To map the epitope recognized by each of the monoclonal antibodies, hybridoma supernatants were screened against lysates from *E. coli* expressing GST or GST::Cfp4 fusion-expressing bacteria (Fig. 2D).
blotting results (Fig. 2D) indicate that 2D20 and 2F9 both recognize epitope 2 (Cfp4 fragments contained in pCR617 and pCR619 but not pCR618), whereas the 3G14 hybridoma produces an antibody that is specific for epitope 5 (Cfp4 fragments contained in pCR618 but not pCR617 and pCR619). The recognition of epitope 2 by 2D20 and 2F9 is qualitatively different, suggesting that the antibodies produced by these two hybridomas are not identical.

Previously, we determined that the Cfp4 protein was highly modified by N-linked glycosylation (19). Glycosylation is characteristic of many extracellular proteins that transit through the general secretory pathway. In particular, N-linked glycosylation substantially alters the Cfp4 protein migration in one-dimensional denaturing acrylamide gels; glycylated Cfp4 migrates variably as a high-molecular-mass diffuse smear, whereas deglycosylation of Cfp4 by PNGase F treatment increases the mobility of Cfp4 as a specific band near 33 kDa (see Fig. S2 in the supplemental material). Even after removal of N-linked glycans through the N-linked-specific deglycosylase PNGase F, Cfp4 does not migrate with the predicted molecular mass, implying additional posttranslational modifications (e.g., O-linked glycosylation).

To determine which asparagine residues serve as sites for N-linked glycosylation, we used site-directed mutagenesis to eliminate candidate positions for glycan attachment. Between the three major phylogenetic lineages (NAm1, NAm2, and Panama), the respective Cfp4 proteins have 80 to 85% amino acid identity, with most variation occurring in the proline/threonine-rich region (amino acids 40 to 67) that follows the signal peptide (Fig. 3A). The sequon beginning with the asparagine (NAm1) or the serine/threonine residue of the consensus site (NAm2), and was not considered further. The thromine residues in the proline/threonine-rich region of amino acids may serve as the site for O-linked glycosylation. Bioinformatic examination of the Cfp4 amino acid sequence N-glycan attachment prediction algorithms identified 7 asparagine residues for potential N-linked glycan modification, including four with the consensus N-linked glycosylation sequon Asn-X-Ser or Asn-X-Thr (Fig. 3A). The sequon beginning with the asparagine at amino acid 147 was not conserved in all strains, missing either the asparagine (NAm1) or the serine/threonine residue of the consensus site (NAm2), and was not considered further. The remaining three sites, N69, N115, and N134, were considered potential sites for N-linked glycan attachment to Cfp4.

Candidate asparagines were mutated to alanine singly and in combination, and the transgenes were expressed in the cfp4 mutant background (strain OSU6). N-linked glycosylation of Cfp4 prevents the wild-type protein from efficiently entering and migrating through acrylamide gels, whereas Cfp4 without N-linked glycans migrates around 33 kDa. Thus, absence of the Cfp4 mutant protein on immunoblots of culture filtrates without PNGase F treatment was used as an indicator that the mutant Cfp4 proteins still had substantial N-linked glycans. None of the single asparagine-to-alanine mutations permitted migration of Cfp4 through acrylamide gels, consistent with N-linked glycans present at multiple sites (Fig. 3B). Consequently, mutated asparagines were combined as pairs and as a triple mutant protein lacking all three candidate asparagines. Expression of the N69A N115A N134A triple mutant enabled migration of Cfp4 through the acrylamide gel nearly as well as fully deglycosylated Cfp4, indicating that at least two of these residues are sites of substantial glycosylation. The N69A N134A protein as well as the N115A N134A protein did not enter the gel, indicating substantial glycan modification still remaining in these double mutant proteins. On the other hand, the N69A N115A Cfp4 protein migrated into the gel, albeit with slightly slower mobility than the triple N69A N115A N134A mutant protein. Together, these results are consistent with N69 and N115 being sites of large N-glycan modification, and the slower mobility of the N69A N115A mutant protein that retains the N134 site suggests some smaller oligosaccharide attachment to N134.

Cfp4 is a conserved exoantigen. To test for conservation of

![FIG 3](http://iai.asm.org/)

### FIG 3 Identification of N-linked glycosylation modification sites on Cfp4.

(A) Amino acid sequence of Cfp4 from three phylogenetic groups of *Histoplasma* indicating sites of potential N-linked glycosylation. Shading of amino acids represents completely conserved residues (dark shading) or conservation in 2 of 3 lineages (light shading). Consensus N-linked glycosylation sequons (Asn-X-Ser/Thr) are indicated in bold, with the corresponding asparagine residue above the sequence. (B) Delineation of N-linked glycosylation sites on Cfp4 through mutation of candidate sites. Culture filtrates from *Histoplasma* yeasts expressing mutant Cfp4 proteins (strains OSU217 to OSU226) were immunoblotted using the 2D20 antibody. Culture filtrate proteins were electrophoresed without prior treatment with PNGase F (−PNGaseF; top) or with PNGase F treatment (+PNGaseF; bottom). The leftmost and rightmost lanes in both panels contained PNGase F-treated culture filtrate proteins from the wild type [WT (−PNGaseF); G186A strain]. Labels above the panels indicate which specific asparagines were mutated to alamines in the mutant proteins.
Cfp4 protein production and secretion, culture filtrates from laboratory and clinical isolates were screened by immunoblotting for Cfp4. Clinical isolates of *Histoplasma* were typed as either NAm1 or NAm2 by exploiting PCR size polymorphisms of the YP53 gene and PCR-restriction fragment length polymorphisms (RFLP) in the *SOD3* gene (see Fig. S3 in the supplemental material). Culture filtrates from two NAm1 isolates (Hc06 and Hc20) were tested, as well as six NAm2 isolates (Hc01, Hc10, Hc16, Hc22, Hc27, and G217B) and the G186A Panama lineage strain. Immunoblots of the culture filtrate proteins showed that all strains secreted Cfp4 protein (Fig. 4), indicating that Cfp4 is a conserved extracellular factor of *Histoplasma* yeasts. The Cfp4 protein from NAm1 isolates showed slightly slower mobility than the Cfp4 of NAm2 isolates after removal of N-linked glycans. Although the NAm1 Cfp4 protein is 5 amino acids larger than the NAm2 Cfp4 protein, this difference is unlikely to be the sole contributor to the greater-than-1-kDa shift observed. This may suggest the Cfp4 of NAm1 *Histoplasma* has a higher degree of O-linked glycan modification than that of NAm2, since the O-linked glycans are not affected by PNGase F treatment.

Given the conserved production of Cfp4 by *Histoplasma* strains, we evaluated the possible utility of Cfp4 as a diagnostic immunoreactive antigen. Previous results indicated that the Cfp4 gene is transcribed during mouse lung infection (19). To test for Cfp4 production during human infection, culture filtrates derived from G186A and G217B background wild-type and cfp4 mutant strains were probed with immune sera from eight human patients with confirmed *Histoplasma* exposure by complement fixation tests. As the sera were obtained from an outbreak investigation, the cases were likely acute or subacute pulmonary histoplasmosis. Without PNGase F treatment, a few background bands were observed, but few differences were noted between Cfp4-proficient and Cfp4-lacking culture filtrates. However, after PNGase F treatment of the *Histoplasma*-secreted proteins, human immune sera strongly recognized a Histoplasma protein around 33 kDa (Fig. 5). The identity of this immunoreactive protein was Cfp4, since no 33-kDa protein was detected in culture filtrates harvested from Cfp4-deficient strains. The human immune sera recognized both the G217B (NAm2) Cfp4 and the G186A (Panama) Cfp4 despite all patients being from the United States and not exposed to the Panama *Histoplasma* lineage (data not shown). At a qualitative level, no particular correlation was observed between Cfp4 reactivity by immunoblotting and complement fixation titers (Fig. 5). Normal (healthy) human sera did not react with the Cfp4 protein, nor did pooled immune sera from patients infected with the related fungus *Blastomyces dermatitidis* (Fig. 5). The strong immunoreactivity of the Cfp4 protein with histoplasmosis immune sera indicates Cfp4 has potential as a diagnostic antigen for human histoplasmosis.

**Cfp4 is not essential for Histoplasma pathogenesis.** The conserved production, abundant secretion, and pathogenic yeast-phase-specific expression of Cfp4 suggests that Cfp4 could contribute to *Histoplasma* pathogenesis. Since macrophages are the primary host cell for *Histoplasma* yeasts, we investigated whether Cfp4 was required for macrophage infection. To test for Cfp4 function in macrophage infection and lysis, wild-type and Cfp4-

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**FIG 4** Secretion of Cfp4 protein by three divergent phylogenetic lineages of *Histoplasma* yeasts. Culture filtrates collected from NAm1 strains (Hc06 and Hc20), NAm2 strains (Hc01, Hc10, Hc16, Hc22, Hc27, G217B), and the Panama strain (G186A) were treated with PNGase, and the proteins were separated by one-dimensional SDS-PAGE followed by immunoblotting with 2D20 and 2F9 antibodies.

**FIG 5** Immunoreactivity of Cfp4 in humans with histoplasmosis. Immunoblots of culture filtrates collected from Cfp4-producing (CFP4; OSU37) and Cfp4-depleted (CFP4-RNAi; OSU87) *Histoplasma* yeasts using sera from patients with histoplasmosis, healthy individuals (normal human sera [NHS] from residents of Indiana), and pooled high-titer sera from patients with blastomycosis. Culture filtrate proteins (500 ng total protein) were treated with PNGase F and separated by electrophoresis for immunoblotting. The yeast-histoplasmin and mycelial-histoplasmin complement fixation titers (CF-Y and CF-M, respectively) of individual histoplasmosis patient serum are listed below each immunoblot. "nd" indicates no detection by complement fixation tests.
Cfp4-producing (OSU45 and OSU37) and Cfp4-lacking (OSU84 and OSU87) yeasts caused lysis of 70 to 75% of the macrophages relative to macrophage monolayers without *Histoplasma* infection. In contrast, macrophages infected with a virulence-attenuated strain due to loss of α-(1,3)-glucan caused lysis of only 30% of the macrophages. No deficiency in macrophage killing was found between wild-type and *cfp4* mutant yeast from either the G186A (Panama) or the G217B (NAm 2) background.

Although *Histoplasma*-dependent lysis of host cells was equivalent with and without Cfp4, Cfp4 could function in enhancing *Histoplasma*’s ability to infect and survive within macrophages. To test this, Cfp4-producing and Cfp4-deficient yeasts were used to infect human primary macrophages derived from peripheral blood monocytes. We observed no reduced survival of Cfp4-deficient yeasts in nonactivated macrophages (data not shown). To increase the stringency of this test, experiments were repeated with macrophages that were activated by treatment with IFN-γ prior to infection. Enumeration of intracellular yeasts showed no differences in survival and replication between the Cfp4-producing (OSU45 and OSU37) and Cfp4-deficient (OSU84 and OSU87) strains (Fig. 6B). This survival of Cfp4-deficient yeasts is in contrast to yeasts that lack the Sod3 extracellular superoxide dismutase, which has reduced survival in activated macrophages (Fig. 6B). Thus, Cfp4 is not essential for infection of and survival within human macrophages.

Although Cfp4 does not appear to function in the interaction of *Histoplasma* with macrophages in culture, we used Cfp4-producing (OSU45) and Cfp4-deficient (OSU84) strains to establish sublethal respiratory infections in mice to address any potential role of Cfp4 in *Histoplasma* virulence in vivo. After intranasal infection, both the wild-type and *cfp4* mutant *Histoplasma* yeasts exhibited similar infection kinetics in lung tissue with rising fungal burdens through day 12 and afterward declining with the onset of cell-mediated immunity (Fig. 7A and B). The kinetics and fungal burden in spleen tissue were also identical for mice infected with wild-type and *cfp4* mutant strains, indicating that Cfp4 was not required for extrapulmonary dissemination of the infection (Fig. 7C and D). As differences exist between the virulence factor production and requirement between *Histoplasma* phylogenetic groups (35–37), we tested whether Cfp4 was required by NAm2 *Histoplasma* using the *gfp*-RNAi and *cfp4*-RNAi lines of the G217B background (OSU37 and OSU87, respectively). Similar to the results with the G186A background, lung infection kinetics and fungal burdens were similar regardless of the production of Cfp4 (Fig. 8A and B). In the G217B background, a 5-fold reduction in spleen fungal burden was observed at day 8 and was also decreased at day 12 in the absence of Cfp4 (Fig. 8C and D). This was consistent through multiple mouse infections. Although statistically significant, this Cfp4-dependent difference in virulence is transient, and after day 16, identical fungal burdens are found in the spleens of mice infected with Cfp4-producing and Cfp4-lacking *Histoplasma* yeasts.

As a more sensitive test of the potential contribution of Cfp4 to *Histoplasma* pathogenesis, we competed Cfp4-producing and Cfp4-deficient strains in vivo. To enable separate enumeration of Cfp4-producing yeast and Cfp4-deficient yeast after coinfection of the same mouse, we marked the Cfp4-producing yeast with the td-Tomato red fluorescent protein (RFP) and Cfp4-deficient yeasts with green fluorescent protein (GFP). To compute the relative fitness (i.e., competitive index), equal amounts of the two strains (OSU77 and OSU85) were used to inoculate mice and

![FIG 6](http://iai.asm.org) Cfp4 production is not essential for Panama or NAm2 lineage *Histoplasma* virulence in macrophages. (A) Determination of macrophage killing ability of yeasts with and without Cfp4. P388D1 macrophages expressing LacZ were infected with *Histoplasma* yeasts at a multiplicity of infection (MOI) of 1:1. After 7 days of coculture, the relative survival of macrophages was determined by quantifying the remaining macrophage-produced LacZ activity. As controls, macrophages were left uninfected or were infected with the attenuated *Histoplasma* strain lacking α-glucan (*gfpΔ*: strain OSU18), which is deficient in macrophage killing. (B) *Histoplasma* yeast survival in IFN-γ-activated human macrophages with and without Cfp4. Human macrophages were derived from peripheral blood monocytes, activated with IFN-γ, and infected with *Histoplasma* yeasts at an MOI of 1:50. Yeast survival was determined by enumeration of viable CFU after 4 h of cocubcation at 37°C. Results are plotted as survival relative to yeasts incubated in the absence of macrophages. In all experiments, the data represent the means ± standard deviations (n = 3 replicates). All macrophage infections were conducted using Cfp4-producing yeasts (*Cfp4*; strain OSU45) or *cfp4* mutant yeasts (*cfp4::T-DNA*: strain OSU48) of the G186A (Panama) background or Cfp4-producing yeasts (*gfp-RNAi*: strain OSU37) or Cfp4-depleted yeasts (*Cfp4-RNAi*: strain OSU87) of the G217B (NAm2) background. The superoxide dismutase-deficient sodΔ strain (OSU15) was used as a control for attenuated survival in macrophages. No statistically significant differences were detected (Student’s t test) between infections with Cfp4-producing and Cfp4-deficient strains.

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liquid broth, and the resulting proliferation in both environments was determined. The growths of both Cfp4-producing and Cfp4-deficient yeasts were nearly identical in liquid broth culture (competitive index of 0.8) (Fig. 9), indicating no role for Cfp4 during growth in vitro. After 9 days of lung infection, the competitive index was 0.8, also indicating Cfp4 confers no significant fitness advantage in vivo. These in vivo fitness data indicate that Cfp4 is dispensable for acute histoplasmosis.

**FIG 7** Cfp4 is not essential for Panama lineage *Histoplasma* yeasts' ability to cause acute histoplasmosis in mice. Virulence of G186A background yeasts in vivo as assessed by fungal burdens in lungs (A and B) and spleens (C and D) following sublethal infection. Mice were infected intranasally with Cfp4-producing (CFP4; strain OSU45) or Cfp4-lacking (cfp4::T-DNA; strain OSU84) yeasts, and the fungal burden in tissues was determined by plating of the tissue homogenates and enumeration of viable CFU at 4-day increments postinfection. Data represent the fungal burdens in individual mice, and horizontal bars indicate the average burden. Dashed horizontal line in lung graphs indicates the inoculum delivered. Fungal burdens at each time point between Cfp4-producing and cfp4 mutant strains were compared by Student’s t test, but no data points had statistical significance (P < 0.05).

**FIG 8** Cfp4 is not essential for NAm2 lineage *Histoplasma* yeasts' ability to cause acute histoplasmosis in mice. Virulence of G217B background yeasts in vivo as assessed by fungal burdens in lungs (A and B) and spleens (C and D) following sublethal infection. Mice were infected intranasally with Cfp4-producing (gfp-RNAi; strain OSU37) or Cfp4-lacking (CFP4-RNAi; strain OSU87) yeasts, and the fungal burden in tissues was determined by plating of the tissue homogenates and enumeration of viable CFU at 4-day increments postinfection. Data represent the fungal burdens in individual mice, and horizontal bars indicate the average burden. Dashed horizontal line in lung graphs indicates the inoculum delivered. Fungal burdens at each time point between Cfp4-producing and cfp4 mutant strains were compared by Student’s t test, and statistical significance is indicated by asterisks (*, P < 0.05; **, P < 0.01).
Discussions

Virulence is a unique feature of the yeast phase of *Histoplasma*, and nearly all demonstrated virulence factors exhibit preferential intracellular localization, and conserved production among three separate phylogenetic groups. Despite these similarities, our data with Cfp4-depleted strains indicate that Cfp4 is dispensable for acute infection, dilutions of the yeast suspensions or the lung homogenates were plated and compared to plate counts of the inoculum mixture. The proportions of Cfp4-producing and Cfp4-deficient yeasts were determined as green and red fluorescent colonies, respectively. The competitive index was calculated as the GFP/RFP ratio at the endpoint divided by the GFP/RFP ratio of the inoculum. Horizontal lines and error bars represent the mean competitive index ± standard deviation (n = 4 replicates [in vitro] or infected mice [in vivo]). The competitive index did not differ significantly from 1.0 for in vitro (P = 0.51) or in vivo (P = 0.58) growth, as determined by Student’s t test. Additionally, no significant difference (P = 0.88) was found between in vivo and in vitro growth.

**FIG 9** Cfp4 does not confer a competitive advantage to *Histoplasma* during murine infection. Competitive index determination of Cfp4-producing and Cfp4-deficient yeasts in vitro and in vivo. Equivalent proportions of Cfp4-producing yeasts marked with GFP (strain OSU777) and Cfp4-lacking yeasts marked with RFP (strain OSU85) were combined and used to inoculate liquid medium (broth culture) and for intranasal infection of mice (inoculum of 10⁴ total yeast cells). After 4 days of growth in broth culture and 9 days of lung infection, dilutions of the yeast suspensions or the lung homogenates were plated and compared to plate counts of the inoculum mixture. The proportions of Cfp4-producing and Cfp4-deficient yeasts were determined as green and red fluorescent colonies, respectively. The competitive index was calculated as the GFP/RFP ratio at the endpoint divided by the GFP/RFP ratio of the inoculum. Horizontal lines and error bars represent the mean competitive index ± standard deviation (n = 4 replicates [in vitro] or infected mice [in vivo]). The competitive index did not differ significantly from 1.0 for in vitro (P = 0.51) or in vivo (P = 0.58) growth, as determined by Student’s t test. Additionally, no significant difference (P = 0.88) was found between in vivo and in vitro growth.

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