Disparate Proteome Responses of Pathogenic and Nonpathogenic Aspergilli to Human Serum Measured by Activity-Based Protein Profiling (ABPP)*

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Aspergillus fumigatus is the primary pathogen causing the devastating pulmonary disease Invasive Aspergillosis in immunocompromised individuals. There is high genomic synteny between A. fumigatus and closely related rarely pathogenic Neosartorya fischeri and Aspergillus clavatus genomes. We applied activity-based protein profiling to compare unique or overexpressed activity-based probe-reactive proteins of all three fungi over time in minimal media growth and in response to human serum. We found 360 probe-reactive proteins exclusive to A. fumigatus, including known virulence associated proteins, and 13 proteins associated with stress response exclusive to A. fumigatus culture in serum. Though the fungi are highly orthologous, A. fumigatus has a significantly greater number of ABP-reactive proteins across varied biological process. Only 50% of expected orthologs of measured A. fumigatus reactive proteins were observed in N. fischeri and A. clavatus. Activity-based protein profiling identified a number of processes that were induced by human serum in A. fumigatus relative to N. fischeri and A. clavatus. These included actin organization and assembly, transport, and fatty acid, cell membrane, and cell wall synthesis. Additionally, signaling proteins regulating vegetative growth, conidiation, and cell wall integrity, required for appropriate cellular response to external stimuli, had higher activity-based probe-protein reaction over time in A. fumigatus and N. fischeri, but not in A. clavatus. Together, we show that measured proteins and physiological processes identified solely or significantly over-represented in A. fumigatus reveal a unique adaptive response to human protein not found in closely related, but rarely pathogenic aspergilli. These unique activity-based probe-protein responses to culture condition may reveal how A. fumigatus initiates pulmonary invasion leading to Invasive Aspergillosis. Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.026534, 1791–1805, 2013.

Invasive aspergillosis (IA)¹ is a devastating infection caused by the ubiquitous saprophytic filamentous fungus Aspergillus fumigatus (Af) (1). Af is an opportunistic pathogen with no true virulence factors. Its pathogenicity is often attributed to its thermotolerance, response to oxidative stress, ability to grow in hypoxic or iron limiting environments, and its ability to use a variety of carbon and nitrogen sources as nutrients, such as proteins derived from the human host (2). A thorough understanding of biological processes or factors that facilitate pathogenic Af infection compared with other microbial infections is needed to assist treatment and diagnosis of IA.

Af protein activity regulation and function, attenuated by environmental response and adaptation, is critical for opportunistic infection and development of IA (3). Activity-based protein profiling (ABPP), coupled to mass spectrometry (MS), is a powerful chemical biology approach for directly identifying a subset of proteins (4). ABPP employs activity-based probes (ABPs) to covalently label and enrich functional families of proteins, thereby reducing the complexity of a proteome, and facilitating observation of low abundance proteins (4). The approach is uniquely suited for studying the ABP-reactive proteome response to human protein by Af when compared with related, but rarely pathogenic Neosartorya fischeri (Nf) and Aspergillus clavatus (Ac).

In a recent study, we used ABPP in combination with a MS-based approach to investigate ABP-protein reactivity of Af (5). We multiplexed, for simultaneous measurement, our click chemistry compatible general cysteine reactive vinyl sulfonate ester (VSE-1) and serine hydrolase specific fluorophospho-

¹The abbreviations used are: IA, invasive aspergillosis; Af, Aspergillus fumigatus; Nf, Neosartorya fischeri; Ac, Aspergillus clavatus; ABPP, activity based protein profiling; ABP, activity-based probe; VSE, vinyl sulfonate ester; Fs, fluorophosphonate; HS, human serum; CC, click chemistry; AMT, accurate mass and time.

The SEQUEST search results for all biological replicates and culture conditions have been deposited in the Biological MS Data and Software Distribution Center data repository under publication number 1070. The data can be accessed at http://omics.pnl.gov/view/publication_1070.html.
phanate (Fp-2) ABPs to analyze the ABP-reactive Af proteome of mycelia cultured in the presence and absence of human serum (HS) (5). Comparison of probe-labeled samples to nonprobe labeled global samples revealed that VSE-1 and Fp-2 enriched a defined subset of proteins. Hence, we identified a series of distinct responses of Af to HS.

Herein, we hypothesize that Af uniquely reacts to HS when compared with rarely pathogenic Nf and Ac (6–9). Fedorova et al. found that the three species share a core of 7514 genes, and that only 8.5%, 13.5%, and 12.6% of the Af, Nf, and Ac genomes are organism specific (6). The high percentage of genomic overlap, which includes virulence associated genes, suggests that IA infection may be caused by protein regulation and function in response to environment (10, 11). Therefore, we have employed ABPP coupled to MS to identify ABP-reactive proteins and associated pathways in Af dramatically over- or under-represented in the other aspergilli in the presence of HS. We believe that unique and/or overexpressed functional pathways may be the true virulence “factor” of Af, by enabling adaptation to environmental stress and deployment of requisite metabolic regulation to survive and replicate in the host environment.

**MATERIALS AND METHODS**

**Strains, Media, and Culture Conditions**—N. fischeri ATCC® MYA-1020™ and A. clavatus ATCC® MYA-1007™ were obtained from ATCC. PDA plates were inoculated with conidia from glycerol stocks, and the conidia were counted by hemocytometer. For liquid culture, complete minimal media (AMM: 1% glucose, 2 ml/L Hunter’s trace elements)(12) with or without 10% HS was grown at 30 °C (Af) and agitated at 150 rpm on an orbital shaker for 24 h or 48 h. Liquid culture, complete Aspergillus minimal media (AMM: 1% glucose, 2 ml/L Hunter’s trace elements)(12) with or without 10% HS was inoculated to a final concentration of 1 × 10^6 conidia/ml. Biomass was grown at 30 °C (Ac) or 37 °C (Nf) and agitated at 150 rpm on an orbital shaker for 24 h or 48 h. Humann Serious—(HS Sigma) was stored at −20 °C and used without further processing.

**Growth Curve**—AMM and AMM containing 10% HS were inoculated to 1 × 10^6 conidia/ml with Nf or Ac and cultured as described above. Fungal biomass was harvested in quadruplicate at each time point by filtering the liquid culture through Miracloth, washing the collected biomass with water, and freeze-dried. The mass of the dried biomass was calculated via decoy database searches, and assembled into two technical replicates for a total of eight runs per sample type (fungus/condition/time point). All samples, including Af (5), were blocked and randomized to minimize instrument and column bias. Identified protein abundances and abundances for each growth condition were analyzed by LC-MS before normalization before LC-MS analysis.

**Cell Lysis**—Biomass was harvested at 24 h and 48 h, passed through Miracloth to remove spent media, and the retained biomass was washed with 15 ml PBS (1×, pH 7.4; 11.9 mM phosphates, 137 mM NaCl, 2.7 mM KCl). Excess media was squeezed out of the biomass. The biomass was manually cut then lysed with a Bullet Blender (NextAdvance): 0.55 mm zirconium silicate beads (0.5–1.0 ml) and PBS (1–2 vols) per 5 ml tube. Lysed samples were centrifuged at 3500 × g for 5 min, pellets were washed with PBS (1 ml) and spun at 3500 × g for 5 min, and the combined supernatants were pelleted twice more. Sonication of the final combined supernatant provided global cell lysate (gcl). The gcl were flash frozen in liquid nitrogen and stored at −80 °C until experimental use. All material was generated such that there were four biological replicates per sample type (fungus/condition/time point).

In **vitro** Probe Labeling for Mass Spectrometry Measurement—Fungal mycelia gcl proteomes (in PBS) from each biological replicate were treated with VSE-1 (20 μM) and Fp-2 (200 μM) in a 1.5 ml vial and incubated for 1 h at 37 °C. Following probe incubation, proteomes were treated with click chemistry (CC) reagents to append biotin as we previously reported. After CC, protein concentration was determined with BCA assay and samples were normalized to 230 μg/ml and then enriched as previously described (5). Normalization before biotin/streptavidin enrichment ensures the amount of protein analyzed by LC-MS is dependent on capture of probe-labeled proteins and hence the overall probe-labeling of a sample. After affinity enrichment, peptides were obtained by treating the resin with trypsin (2 μl; trypsin was reconstituted in 40 μl of NH4HCO3 (25 mM, pH 8) and NH4HCO3 (200 μl) and subsequent incubation at 37 °C, 1200 rpm for 15 h. Following digestion, peptide supernatant was obtained (6,000 × g) and the pellet was washed with NH4HCO3 (150 μl). The combined peptide supernatant was dried by speed vacuum, reconstituted in NH4HCO3 (40 μl) and heated for 10 min at 37 °C. The samples were centrifuged at 100,000 × g for 20 min at 4 °C, and 25 μl was collected for MS analysis.

**LC-MS and Data Analysis of ABP-Labeled Proteins**—Four biological replicates per growth condition were analyzed as two technical replicates for a total of eight runs per sample type (fungus/condition/time point). All samples, including Af (5), were blocked and randomized to minimize instrument and column bias. Peptide identifications were filtered with a MS-GF spectral probability score of ≤ 1 × 10^{-5} (16) with a PSM level FDR of <6% calculated via decoy database searches, and assembled into Nf and Ac-specific AMT tag databases. Nf and Ac SEQUEST peptide scores for database generation are available in supplemental Table S1.

All samples with added human serum were searched against the H_sapiens_Uniprot_SPROT fasta (4/5/2011) using SEQUEST as described above. Peptides matching to more than one protein, contaminant porcine trypsin peptides, and peptides not measured in 9 of 16 data sets (Ac) or 8 of 14 data sets (Nf) were removed resulting in 61 peptides and 70 peptides respectively. This list provided five proteins identified by > 1 peptide for Ac and seven proteins for Nf. Of these proteins only one, APOA1_HUMAN identified by two peptides in Nf samples only, may have human serum origin.

For identification and quantification, VIPER (3.49, January 27, 2012) was used to correlate each AMT tag entry with a unique LC-MS feature relying on high mass measurement accuracy (MMA_median ±0.528 ppm and ±0.581 ppm for Nf and Ac respectively) and normalized LC elution time accuracy (NET_median ±0.445% and 0.458% for Nf and Ac respectively) and a 1 ppm error in at least 1 data set (17). The Nf MS data consisted of 7330 peptides across 26 LC-MS data sets; Ac had 6187 peptides across 30 LC-MS data sets, and Af, obtained from Wiedner et al., had 12,018 peptides across 29 LC-MS data sets (5); data sets with low total sum protein abundances by fungus had been removed. The log_{10} peak intensity values (i.e. abundances) for the final peptide identifications were processed in a series of steps using MatLab R2011b that included quality control, normalization, protein quantification, and comparative statistical analyses.
Quality control processing was performed to identify and remove contaminant proteins, redundant peptides, and peptides with an insufficient amount of data across the set of samples (18, 19). Application of confidence metrics resulted in 5378 peptide identifications across 20 Nf data sets, 4936 peptides across 28 Ac data sets, and 10, 028 peptides across 29 Af data sets (supplemental Table S2). Peptides were normalized using a statistical procedure for the analysis of proteomic normalization strategies (SPANS) that identifies the peptide selection method and data scaling factor that minimizes introduction of bias in the data set (20). The peptide abundance values within each data set were normalized to standard regression coefficients computed from a base set of rank invariant peptides with low data content, or were outside the dominant significance pattern (22).

Peptide roll-up and removal of single-peptide identification resulted in patterns that were redundant, had low developed and applied ‘R-Rollup’ method using the most abundant reference peptide, after filtering the peptides that were redundant, had low data content, or were outside the dominant significance pattern (22). Protein roll-up and removal of single-peptide identification resulted in 498, 452, and 888 proteins in Nf, Ac, and Af, respectively. Comparative statistical analyses of time-matched samples were performed using a t test to assess differences in protein average abundance, and a g-test to assess associations among factors because of the presence or absence of HS response. Statistical analysis across time was also performed within a serum category. The protein statistical analysis results can be found in supplemental Tables S3–S5.

For further analysis, proteins were divided into three categories: (1) increased probe-reactivity induced by the presence of HS, (2) reduced probe-reactivity in the presence of HS, and (3) no change in probe reactive patterns in the presence of HS when compared with AMM culture. Protein identifications were removed from the list if fewer than two peptides per protein were found, and if proteins were not observed in two or more biological replicates for at least one culture condition (±HS; 24 or 48 h). For proteins with no change in probe reactive patterns between culture conditions, proteins not observed in two or more biological replicates for both culture conditions were removed. Orthologous proteins between the three species were found using the Ensembl Biomart tool (supplemental Table S6) (23). Proteins were mapped to function and Biological Process GO terms using tools from the Aspergillus Genome Database (24), the FungiFun tools at Omnipfug (25) and the KEGG pathway User mapping tool (26) (supplemental Table S7). GO slims were mapped and counted using the CateGOrizer tool (27).

RESULTS

Fungal Growth Capacity in HS-Supplemented Culture—Aspergillus fumigatus (Af) has the ability to grow in the presence of human serum. Af can sequester iron from transferrin in human serum (28, 29), and human albumin facilitates germination and growth (28, 30). To assess the ability of the nonpathogenic species to grow in the presence of HS, we grew Nf and Ac in aspergillus minimal media (AMM) supplemented with 10% HS and monitored biomass generation (Fig. 1A). Growth in AMM with no added serum was used as a base-line comparison (Fig. 1B). Nf grew as small spherical pellets in the presence and absence of HS, while Ac grew as spherical pellets in AMM, but in the filamentous form in HS culture. Both pellet and filamentous morphological forms involve polarized hyphal extension leading to environmental exploration for nutrients (31, 32). Filamentous versus spherical pellet growth is influenced by factors such as pH, medium composition, inoculum concentration, agitation, and fungal strain (32, 33). Given consistent culture conditions were maintained, except for presence/absence of HS, the filamentous growth of Ac must depend on other unknown factors. Ac is a more distant relative to Af than Nf and it appears to respond differently to HS.

The growth curves of Nf and Ac closely resembled that of Af grown in HS (5). At 24 h of culture, all three aspergilli were in mid-log phase, but by 48 h of growth all three were in stationary or declining phase (Fig. 1A). The growth curves generated from HS culture were quite different from growth in AMM only. In AMM culture (Fig. 1B), all three aspergilli were in early log phase at 24 h of culture, Nf had reached stationary phase by 48 h, Ac growth appeared to slow, and Af was in late log phase growth. When compared with AMM culture, more biomass was obtained for each fungus in HS culture. Additionally, Af produced a greater maximum biomass than Nf and Ac under both conditions, yielding 4.5 mg of biomass per ml of culture in AMM and 5.8 mg of biomass per ml of culture in AMM + 10% HS (5).

ABPP of Aspergilli Global Cell Lysate—We utilized a general cysteine reactive vinyl sulfonate ester ABP (VSE-1), and a
ABPP of Pathogenic and Nonpathogenic Fungi

Fig. 2. ABPP of three aspergilli. A, Activity-Based Probes (ABPs) used for targeting the reactive proteome of each fungi. Probe VSE-1 contains a general cysteine reactive vinyl sulfonate ester. Probe Fp-2 contains the serine hydrolase selective fluorophosphonate reactive group. The incorporation of the alkyne in both ABPs allows simultaneous bio-orthogonal conjugation of a reporter group via a copper-catalyzed cycloaddition (click chemistry). B, Components of an ABP include the reactive group (RG) that covalently binds the protein in an active site directed manner and the Tag. Our ABPs incorporate an alkyne clickable tag for conjugation of reporter groups after protein labeling. C, Experimental design for comparison of reactivity of Af, Nf, and Ac. For all three, the fungus was cultured with and without HS, and biomass was harvested at 24 and 48 h. Global cell lysates of biomass for each condition were treated individually with a mixture of VSE-1 and Fp-2 (i); after probe labeling the biotin reporter group was appended using copper-catalyzed click chemistry (ii), and probe labeled proteins were collected using affinity enrichment and digested on-bead with trypsin for LC-MS/MS analysis (iii). Probe reactive protein lists were then compared between fungi and culture condition based on gene orthology.

serine hydrolase specific fluorophosphonate ABP (Fp-2) (Fig. 2A) (5), to label proteins in Nf and Ac global cell lysates obtained from mycelia grown in the presence and absence of 10% HS at 24 h and 48 h of culture. In previous work, these probes provided an extensive list of probe-reactive proteins across multiple protein and enzyme families thereby providing a broad profile of sample dependent probe-protein reactivity (5). We found by 1D SDS-PAGE fluorescent imaging that ABP-protein reactivity is reduced after 48 h of culture in Af (5). Both ABPs consist of a electrophilic reactive group, and an alkyne for appending reporter groups, such as biotin, via copper catalyzed [3 + 2] cycloadditions (aka., click chemistry (CC)) (Fig. 2B) (4). To label multiple protein families in one sample and to reduce sample number, ABPs VSE-1 and Fp-2 were used simultaneously in a multiplexed fashion. Labeled samples were then analyzed by LC-MS as described in Materials and Methods (Fig. 2C). Previous comparison of a global proteomics and an ABPP analysis of Af culture showed that protein enrichment in ABPP studies is not dependent on protein abundance (5). Presence of a protein in our analysis is directly dependent on its reactivity with either ABP VSE-1 or Fp-2. Therefore, the measured proteins are defined as ABP-reactive proteins. Furthermore, differential protein abundance, which is dependent on ABP-protein labeling, is called ABP-protein reactivity.

ABPP Measured Proteins—Protein reactivity toward ABPs VSE-1 and Fp-2 was greatest in Af regardless of growth condition (Fig. 3). In the presence of HS, 673 and 777 Af proteins were observed at 24 h and 48 h, respectively. In AMM, 782 and 855 Af proteins were observed at 24 h and 48 h. This is consistent with our previous analysis of the Af data, which showed a >10% increase in ABP-reactive proteins in AMM culture (5). Fewer ABP-reactive proteins were identified in Nf, with 352 and 498 proteins measured at 24 h
ABPP of Pathogenic and Nonpathogenic Fungi

**Probe-Reactive Proteins Unique to Af—Comparative genomic analysis of the three aspergilli found 7514 orthologous genes between the three species (6). However, as our results show, the presence of orthologous genes does not directly translate into orthogonal measurement of ABP-reactive proteins. A total of 360 proteins were exclusively ABP-reactive in Af. All but five proteins have orthologous genes in Nf and Ac, but the remaining 355 proteins were not measured and therefore are not probe-reactive in Nf and Ac. The majority of these uniquely reactive proteins were measured regardless of culture condition (288 were observed in ≥ 3 sample types), and therefore ABP-protein reactivity doesn’t appear to be enhanced by the presence of HS (supplemental Table S6).

Within this uniquely ABP-reactive protein list, we found 7 known Af virulence associated proteins. The seven virulence associated proteins were not observed in either Nf or Ac cultures even though these fungi have orthologous genes (Table I) (10). Included are the Ca\+ calmodulin active phosphatase Calcineurin A (CnaA) (single peptide identification in Nf and Ac supplemental Fig. S1) and \(\alpha,\alpha\)-trehalose phosphate synthase (Tps2) genes essential for cell wall integrity, growth, and pathogenicity; knockout mutants are less virulent in IA mouse models, as previously reported (34, 35). The melanin pigment polyketide synthase (Pksp) produces melanin, which protects Af spores against macrophage induced oxidative stress by efficient scavenging of reactive oxygen species (ROS) (36–38). Catalase 1 and catalase 2 also impart protection against oxidative stress via \(H_2O_2\) catabolism (25). Orotidine 5’-phosphate decarboxylase (PyrG) is part of the de novo UMP biosynthetic pathway, and PyrG knockouts have attenuated virulence in mouse IA infection model, likely because of limitation of uridine/uracil in the lung environment (39). Finally, knockout mutants of the chitin synthase ChsG had reduced virulence compared with wild type and caused increased hyphal branching of Af. In Af, virulence associated proteins were found in both HS supplemented and AMM, with the exception of Pksp, which was only observed in AMM alone. This is consistent with necessity of these proteins for growth, development, and stress response, yet the lack of activity in the rarely pathogenic fungi highlights the importance of their associated pathways to the pathogenicity of Af (10).

Functional analysis of the uniquely ABP-reactive proteins using GO term biological process mapping showed considerable overlap of GO terms between the uniquely ABP-reactive Af proteins and the complete observed protein list of Nf and Ac, with a few key exceptions (for a complete list see supplemental Table S7) (25). As discussed above, known pathogenicity associated proteins were not measured in Nf and Ac. In addition, hydrogen peroxide catabolic processes and glutathione biosynthetic processes were not detected in Nf and Ac analyses. Observed proteins associated with oxidation-reduction processes and response to \(H_2O_2\) included the spore specific catalase CatA (AFUA_6G03890), Cat2 (AFUA_8G01670), Cat1 (AFUA_3G02270), catalase fgaCat (AFUA_2G18030), and fatty acid oxygenase PpoC (AFUA_3G12120), and fatty acid oxidase PpoC (AFUA_3G12120) (25). Glutathione production is involved in maintaining the redox state of the cell; two glutathione biosynthetic proteins were observed: glutamate-cysteine ligase (AFUA_3G13900) and glutathione synthase (AFUA_5G06610) (25). These two proteins are orthologs of the S. cerevisiae GSH1 and GSH2 glutathione biosynthetic proteins, which are induced by oxidative stress (40, 41). The differential detection of these ABP-reactive proteins implies that Af is responding to oxidative stress, while Nf and Ac are not.

**Probe-Reactive Proteins Unique to Af HS Culture—** Further analysis of ABP-reactive proteins focused on HS induced reactivity. Virulence associated proteins were not exclusive to HS culture; however, within our 360 uniquely reactive Af proteins, we found 13 proteins exclusive to 48 h HS culture (Table II). No protein identified was exclusively ABP-reactive in 24 h HS culture. Six of the 48 h HS unique proteins have different expression levels under stress. For example, the 12-oxophytodienoate reductase (AFUA_5G14330) and the alcohol dehydrogenase (AFUA_6G10260) transcripts are induced by exposure to airway epithelia cells (42). In contrast, Major Facilitator Superfamily (MFS) glucose transporter (AFUA_3G14170) was induced by hypoxia in Af (43), but the orthologs in A. nidulans and S. cerevisiae are associated with nutrient depletion.
stress. Amino acid catabolism is also a nutrient limitation stress response (44), and the tyrosine catabolism fumaryl-acetoacetate hydrolase FahA (AFUA_2G04230) was exclusive to 48 h 10% HS culture (45). Additionally, tyrosine degradation has been associated with cell wall stress (46). Allantoicase (AFUA_3G12560) and cytochrome P450 monooxygenase (AFUA_6G02210) transcripts increase on exposure to gliotoxin, an Af metabolite, and voriconazole, an antifungal azole drug, respectively (47, 48). It appears that Af stress response mechanisms are active at 48 h in HS culture. These particular HS induced stress response proteins are absent in Nf and Ac indicating a limited stress response of these fungi to HS induced stationary phase.

Comparison of 10% HS Culture Between Aspergilli—Based on high genetic similarity between Af, Nf, and Ac, we expected to find similar numbers of ABP-reactive proteins between the three fungi, but of the observed Af ABP-reactive proteins, only ~50% were observed in Nf and Ac. The expected number of proteins in Nf and Ac were calculated from the respective proteins orthologous to measured Af proteins. When specifically comparing HS culture conditions across time (24 and 48 h), 667 and 771 protein orthologs were expected in Nf, but only 324 and 426 were observed at 24 and 48 h, respectively (Fig. 4). In Ac samples, 664 and 759 proteins were expected, but 345 and 309 were detected at 24 and 48 h, respectively (Fig. 4). Functional enrichment

### Table I

| Description | Gene name | E. C. | Af | Nf | Ac |
|-------------|-----------|------|----|----|----|
| Orotidine 5′-phosphate decarboxylase | PyrG* | 4.1.1.23 | AFUA_2G08360 | NFIA_083990 | ACLA_080190 |
| Bifunctional catalase-peroxidase | Cat2 | 1.11.1.6, 1.11.1.7 | AFUA_8G01670* | NFIA_095260 | ACLA_044200 |
| Mycelial catalase | Cat1 | 1.11.1.6 | AFUA_3G02270* | NFIA_003430 | ACLA_062020 |
| Chitin synthase | ChsG | 2.4.1.16 | AFUA_3G14420 | NFIA_062840 | ACLA_041870 |
| Calcinurin catalytic subunit | CnaA | 3.1.3.16 | AFUA_5G09360 | NFIA_077970* | ACLA_011200* |
| α,α′-trehalose phosphate synthase | Tps2 | 2.4.1.15 | AFUA_3G05650 | NFIA_071540 | ACLA_033660 |
| Conidial pigment polyketide synthase |Pksp/Alb1 | AFUA_2G1760* | NFIA_093000 | ACLA_076490 |

* Protein essential for growth.  
* Protein not measured in 10% HS 24 h.  
* Protein only measured in AMM 48 h.  
* Protein was measured by 1 peptide (supplemental Fig. S1).

### Table II

| Locus tag | Description | Gene name | Function/process |
|-----------|-------------|-----------|-----------------|
| AFUA_4G08880 | Possible apospory-associated protein c | Unknown function |
| AFUA_2G04230 | Fumarylacetoacetate hydrolase | Degradation of L-tyrosine |
| AFUA_2G10170 | Conserved hypothetical protein | Unknown function |
| AFUA_2G15520 | FAD monooxygenase | Predicted role in oxidation/reduction process |
| AFUA_3G05740 | Aldose 1-epimerase | Response to stress (S. pombe) |
| AFUA_3G10390 | Conserved hypothetical protein | Unknown function |
| AFUA_3G12560 | Allantoicase | Alc | Induced by gliotoxin exposure |
| AFUA_3G14170 | MFS glucose transporter | hxA | Induced by hypoxia |
| AFUA_5G14330 | 12-oxophytodienoate reductase | Cyp548D2 | Induced by exposure to airway epithelial cells |
| AFUA_6G02210 | Cytochrome P450 monooxygenase | AKR1 | Induced by exposure airway epithelial cells |
| AFUA_6G10260 | Aldehyde reductase | Predicted role in oxidation/reduction process |
| AFUA_6G13830 | Oxidoreductase, short chain dehydrogenase/reductase family | Predicted heme binding activity |

* Expected orthologs 24 h, measured proteins 24 h.  
* Expected orthologs 48 h, measured proteins 48 h.  
* Expected orthologs 24 h, measured proteins 48 h.
analysis of the complete list of 24 h and 48 h 10% HS reactive proteins for each organism was performed by mapping protein identity with GO term biological processes (supplemental Table S7). We identified 78, 49, and 63 enriched biological processes for \( A f \), \( Nf \), and \( Ac \) respectively at 24 h HS culture, and 85, 65, and 53 enriched biological processes at 48 h. The greater number of biological process terms in \( A f \) is a reflection of uniquely ABPP identified proteins and the greater number of gcl ABP-reactive proteins as a whole when compared with \( Nf \) and \( Ac \). In order to summarize and compare the enriched terms between species, we mapped the Biological Process GO terms to broad GO slim categories and counted the consolidated single occurrence of each GO slim using the online web tool CateGOrizer (Fig. 5) (27). Many of the GO slim categories are found in all three fungal species with the exception of stress response (\( A f \)), cytoskeleton organization and biogenesis (\( Nf \)), and cell cycle (\( Ac \)) (Fig. 5). The occurrence of GO slims is higher in \( A f \) because of higher numbers of reactive proteins associated with biological processes and GO slims.

Many \( A f \) essential proteins orthologs were measured in \( Nf \) and \( Ac \). Genes essential for fungal growth can be considered targets for antifungal drug development. We found a total of 19 \( A f \) proteins essential for normal growth with ABP-protein reactivity in at least three of four \( A f \) culture conditions (Table III) (49). Three proteins, 1, 3-\( \beta \) glucan synthase Fksp (AFUA_6G12400), GUK1, and the mitochondrial import receptor subunit (Tom40) were exclusive to \( A f \). The protein transport protein Sec31 and glycogen synthase kinase (Skp1) were only measured in \( A f \) and \( Nf \), and the glutamyl tRNA synthetase (GUS1) was not observed in \( Nf \). These \( A f \) proteins are annotated with metabolism, biosynthesis and RNA processing function, and are essential for fungal growth in \( A f \). Overlapping measurement in all three fungi across culture conditions suggests that these proteins may also be essential in \( Nf \) and \( Ac \).

Comparison of Probe Labeling Trends Between Culture Conditions—ABPP yields measurement of protein abundance dependent on ABP labeling and enrichment of ABP-labeled proteins. In our prior ABPP analysis of \( A f \), we validated our ABPP LC-MS based measurements of protein reactivity by coupling our data to functional enzyme assays (5). Herein, we compared ABP-protein reactivity measured at each time point and culture condition for two cross-conditional, absence or presence of HS, and two longitudinal comparisons, 24 h and 48 h, within each fungus (Fig. 6). Protein abundance was normalized as described above, a t test applied to assess significant differences in average ABP-labeled protein abundance between culture conditions, and a g-test applied to assess the presence or absence of response, i.e. proteins
ABPP of Pathogenic and Nonpathogenic Fungi

Table III

| Protein description            | Gene name | Biological process                  | E.C.         | Af locus tag           | Nf locus tag           | Ac locus tag           |
|-------------------------------|-----------|--------------------------------------|--------------|------------------------|------------------------|------------------------|
| 1,3-beta-glucan synthase      | Fksp      | Cell wall organization and biogenesis| 2.4.1.34     | AFUA_6G12400          | NFIA_058360            | ACLA_085650            |
| catalytic subunit             |           |                                      |              |                        |                        |                        |
| Mitochondrial import receptor | tom40     | Protein transport                    | AFUA_6G05110 | NFIA_051680            | ACLA_095430            |                        |
| subunit                       |           |                                      |              |                        |                        |                        |
| Guanylate kinase              | GUK1      | Nucleotide metabolism                | 2.7.4.8      | AFUA_1G08840          | NFIA_016820            | ACLA_026640            |
| Glycogen synthase kinase      | Skp1      | Cell cycle control                   | 2.7.1.-      | AFUA_6G05120          | NFIA_051690            | ACLA_095420            |
| Protein transport protein     | Sec31     | Protein transport                    | AFUA_2G12980 | NFIA_088150            | ACLA_071790            |                        |
| Glutamyl-tRNA synthetase      | GUS1      | Protein translation                  | 6.1.1.17     | AFUA_5G03560          | NFIA_038550            | ACLA_001610            |
| GMP synthase                  | GUA1      | Metabolism                           | 6.3.5.2      | AFUA_3G01110          | NFIA_001970            | ACLA_063690            |
| 60S ribosomal protein         | L17       | Protein biogenesis                   | AFUA_1G14410 | NFIA_011010            | ACLA_021010            |                        |
| Polyadenylate-binding protein | Pab1      | Protein translation                  | AFUA_1G04190 | NFIA_020550            | ACLA_030250            |                        |
| Tubulin alpha-1 subunit       | TUB-1     | Cytoskeleton organization and biogenesis| AFUA_1G02550 | NFIA_022070            | ACLA_031860            |                        |
| Stearic acid desaturase        | SdeA      | Metabolism                           | 1.14.19.1    | AFUA_7G05920          | NFIA_027160            | ACLA_007610            |
| Ribosomal protein             | LT4       | Protein biogenesis                   | AFUA_6G03830 | NFIA_050350            | ACLA_096780            |                        |
| Glucosamine-fructose-6-phosphate amidotransferase | GFA1 | Cell wall organization and biogenesis | 2.6.1.16 | AFUA_6G06340 | NFIA_051960 | ACLA_088110 |
| Inorganic diphosphatase       | IPP1      | Metabolism                           | 3.6.1.1      | AFUA_3G03830          | NFIA_068740            | ACLA_036360            |
| Bifunctional tryptophan       | TRPB      | Amino acid biosynthesis              | 4.2.1.20     | AFUA_2G13250          | NFIA_088420            | ACLA_072050            |
| synthase                      |           |                                      |              |                        |                        |                        |
| Acetyl-CoA acetyltransferase  | ERG10     | Ergosterol biosynthesis              | 2.3.1.9      | AFUA_8G04000          | NFIA_096700            | ACLA_057330            |
| Saccharopine dehydrogenase    | Lys9      | Amino acid biosynthesis              | 1.5.1.10     | AFUA_4G11340          | NFIA_105250            | ACLA_050430            |
| 60S Ribosomal protein         | L11       | Protein biogenesis                   | AFUA_4G07730 | NFIA_108440            | ACLA_047100            |                        |
| 14-Alpha sterol demethylase   | Cyp51a    | Ergosterol biosynthesis              | 1.14.13.70   | AFUA_4G06890          | NFIA_109350            | ACLA_046180            |

Protein not measured in 24 h 10% HS culture.
Protein not measured in 48 h 10% HS culture.

Fig. 6. Cross-conditional and longitudinal comparisons of ABP-labeled protein reactivity. In each comparison a comparative statistical analysis was applied across biological replicates using a t test to assess differences in protein abundance (reactivity) and a g-test to assess the presence or absence of response to the time or condition (uniqueness) a p value < 0.05 for each test was used as a significance cut-off.

unique to that condition. In comparison to AMM, Af has 617 ABP-reactive proteins with lower reactivity, and only 32 with higher reactivity in HS. Nf showed a similar trend as Af with 261 ABP-reactive proteins having lower reactivity, and 15 having higher reactivity in the presence of HS at 24 h. In contrast, Ac had only 5 ABP-reactive proteins with higher reactivity in HS, and 395 with lower reactivity in HS. In HS, >50% of ABP-reactive proteins measured in Af and Nf have higher reactivity later in culture. In contrast, only 29 Ac ABP-reactive proteins had higher reactivity over time in HS. Less than 33% of ABP-reactive proteins had induced reactivity over time in AMM.
Probe Reactive Proteins in 24 h 10% HS Culture—We compared HS induced ABP-reactive proteins to AMM culture at 24 h for all three aspergilli. Each fungus is in mid log-phase growth, indicating the fungi have already adapted to, and are compensating for, the presence of HS. The number of HS induced ABP-reactive proteins between the three aspergilli was different (32, 15, 5 proteins) (Fig. 6). Using orthologous genes, this protein list was reduced to 42 proteins that had HS induced ABP-reactivity in at least one fungus. By comparing the fold change in reactivity between AMM and HS culture, we found that HS did not consistently induce ABP-reactive protein abundance within orthologous proteins across all three organisms at 24 h (Fig. 7). Notably, these processes are not as highly represented, and the measured orthologous proteins do not have the same degree of ABP-protein reactivity change in Nf and Ac. In 24 h 10% HS culture we observed the actin assembly endocytotic proteins Pan1 (AFUA_7G03870), Sla2 (AFUA_3G06140), F-actin capping protein (AFUA_6G10060), and vacuolar dynamin-like VspA (AFUA_5G02360) in Af, but these proteins were not observed or did not have higher ABP-reactivity in Nf or Ac (50, 51). Two other putative dynamin-like GTPases were only measured in Af (AFUA_6G11890 and AFUA_8G02840).

Clustering of the highly reactive proteins in Af showed significant representation of proteins involved in actin assembly, transport, and fatty acid, cell wall, and cell membrane biosynthesis (Fig. 7). Notably, these processes are not as highly represented, and the measured orthologous proteins do not have the same degree of ABP-protein reactivity change in Nf and Ac. In 24 h 10% HS culture we observed the actin assembly endocytotic proteins Pan1 (AFUA_7G03870), Sla2 (AFUA_3G06140), F-actin capping protein (AFUA_6G10060), and vacuolar dynamin-like VspA (AFUA_5G02360) in Af, but these proteins were not observed or did not have higher ABP-reactivity in Nf or Ac (50, 51). Two other putative dynamin-like GTPases were only measured in Af (AFUA_6G11890 and AFUA_8G02840). High abundance of endocytotic proteins suggests that the fungus is actively endocytosing extracellular material from the 10% HS culture. Actin is also required for polarized growth (52); orthologs of the essential S. cerevisiae chaperonin containing T-complex cct8 and cct6 involved in actin organization was measured in Af (AFUA_4G006890 and AFUA_3G006140), F-actin capping protein (AFUA_6G10060), and vacuolar dynamin-like VspA (AFUA_5G02360) in Af, but these proteins were not observed or did not have higher ABP-reactivity in Nf or Ac (50, 51). Two other putative dynamin-like GTPases were only measured in Af (AFUA_6G11890 and AFUA_8G02840).

**Fig. 7.** Log2 ratio of 42 probe reactive proteins induced by HS culture. Log2 ratio was calculated as difference in measured protein abundance across ≥2 biological replicates between 24 h HS culture and 24 h AMM culture. The max Log2 ratio of 5 (red) denotes proteins only measured in HS culture and therefore they have no probe-reactivity in AMM culture. The low end of the scale, Log2 ratio of −5 (blue) denotes proteins only measured in AMM culture and therefore they have no probe-reactivity in HS culture. Gray represents proteins not observed.
ABPP of Pathogenic and Nonpathogenic Fungi

Observed signaling proteins and Fold Change difference over time. Probe reactive signaling proteins were measured in each fungus over time in the presence and absence of 10% HS. FC = log₂ difference in protein abundance; a negative value denotes higher reactivity in AMM culture; a value of 0 denotes no significant difference between samples (p value > 0.05). Single peptide identifications were annotated (Figure S1).

| Gene name | Af peptides | Af FC AMM (48 h/24 h) | Af FC HS (48 h/24 h) | Nf peptides | Nf FC AMM (48 h/24 h) | Nf FC HS (48 h/24 h) | Ac peptides | Ac FC AMM (48 h/24 h) | Ac FC HS (48 h/24 h) |
|-----------|-------------|-----------------------|----------------------|-------------|-----------------------|----------------------|-------------|-----------------------|----------------------|
| cdc42     | 2           | 0                     | 3.7                  | 1           | 0                     | 0                    | 1           | −3.4                  | 0                    |
| MpkA      | 2           | 3.0                   | 3.5                  | 2           | 0                     | 1.6                  | 2           | 0                     | 0                    |
| Rac       | 4           | 0                     | 3.3                  | 3           | −0.9                  | 1.8                  |             |                       |                      |
| RasA      | 2           | 0                     | 3.1                  | 7           | −1.1                  | 1.6                  | 6           | −3.8                  | 0                    |
| mkp6      | 3           | 2.4                   | 2.8                  | 2           | −2.2                  | 0.8                  | 2           | 0                     | 0                    |
| Rho1      | 4           | 1.3                   | 2.6                  | 4           | −0.9                  | 1.3                  | 6           | −2.0                  | 0                    |
| GpaA      | 6           | 1.4                   | 2.5                  | 6           | −1.6                  | 1.0                  | 2           | −2.8                  | −1.5                 |
| SakA      | 1           | 0                     | 0.0                  | 3           | 0                     | 0.86                 | 1           | 0                     | 0                    |
| cnaA      | 4           | 0                     | −0.9                 | 1           | 0                     | 1                   | 0           | 0                    | 0                    |

but was not observed in HS cultures of 

| but was not observed in HS cultures of Nf or Ac. Interestingly, vip1 was found to be an antigen of sera from confirmed IA patients (54), and might provide an opportunity for clinical detection. Protein trafficking is important for cell and organelle biogenesis, and Af had highly reactive transport proteins in 10% HS. Protein trafficking proteins measured include two AAA-ATPases: Rpt4 (AFUA_6G06780) and Rpt6 (AFUA_4G04660), which are involved in endoplasmic reticulum-associated degradation (55), as well as Tom70 (AFUA_2G01660), which facilitates lipid transport into mitochondria (56). Tom70 is closely associated with the essential Tom40 in lipid transport; however, in our current study, Tom40 ABP-reactivity did not change between culture conditions. The S. cerevisiae sec26 ortholog, Coatomer β (AFUA_1G10970), involved in ER to Golgi protein trafficking was also observed to have high ABP-reactivity (57). Although transport was also represented in Nf and Ac, induction of protein reactivity was not consistent across all three fungi.

We clustered fatty acid, cell membrane and cell wall biosynthesis processes into a third group of highly reactive proteins. These three processes are related to each other via lipid signaling and ergosterol binding mediated in part by the kinase Ypk1 (AFUA_2G10620) (58, 59). The acetyl CoA carboxylase (Acc1) catalyzes the first step in fatty acid biosynthesis, and is essential in both A. nidulans (Acc1) and the yeast S. cerevisiae (Acc1) (60, 61); high ABP-reactivity of Acc1 in 10% HS culture was measured in all three aspergilli. Additionally, the putative P450 fatty acid hydroxylase (Cyp505A13), predicted to catalyze the oxidation of fatty acids during fatty acid metabolism (26), and the Δ9-stearic acid desaturase (AFUA_7G05920, SdeA), which catalyzes the oxidation of a fatty acid into an unsaturated fatty acid in A. nidulans (62), were measured. Af had HS induced ABP-reactivity of two ergosterol biosynthesis proteins: (1) farnesyl-diphosphate farnesyltransferase (AFUA_7G01220, Erg9) an IA serum antigen, which catalyzes the coupling of two farnesyl pyrophosphate units into squalene at the start of the biosynthetic pathway (64, 63), and (2) the azole drug target Cyp51a (Erg11) (64). Most notably Fksp, a single copy essential gene involved in cell wall synthesis had higher reactivity in Af HS culture (65), and the Nf and Ac orthologs were not observed. As well as being an antifungal target, Fksp may prove useful in clinical detection of Af as it has no human orthologs (66).

Protein Reactive Signaling Proteins over Time—Increasing evidence suggests environmental response and adaptation are keys to the ability of Af to cause opportunistic infection (5). The growth curves of all three fungi show a significant growth response to HS. We compared ABP-reactivity of signaling cascade proteins, necessary for response to the environment, over time between the three fungi (Table IV). We measured in all three fungi a heterotrimeric G-α protein (AFUA_1G13140) that controls duration of G-protein-coupled receptor signal transduction (67). The RasA GTPase (AFUA_5G11230) essential for regulating polarized growth and asexual development, and the downstream effector Rho1 GTPase Rac (AFUA_3G06300), which also has roles in polarized growth response to reactive oxygen species, were also measured (68, 69). Rac was not measured in Ac. Additionally, potential downstream RasA targets MAPK (AFUA_4G13720 and AFUA_6G12820) and cdc42 (AFUA_2G05740) were detected in all aspergilli (70). Finally, we detected the Rho1 GTPase (AFUA_6G06900), attributed to activating the cell wall integrity MAP kinase module in all fungi (71). Although typically detected in all fungi, these signaling proteins had different reactivity within each fungus over time and culture condition. In Ac regardless of culture medium, signaling ABP-reactive protein abundance decreased over time. In Nf, signaling protein abundance decreased over time in AMM, but increased over time in HS. Finally, with the exception of calcineurin A (cnaA), all signaling proteins had increased ABP-reactivity over time in Af in both culture conditions (Table IV).

DISCUSSION

Comparative genomic analysis of pathogenic and nonpathogenic fungi provides valuable information about genetic traits, which may be responsible for opportunistic infection and the evolution of virulence associated factors. However,
the presence of known virulence associated genes in pathogenic and rarely pathogenic species confounds genomic comparisons. Transcriptomics and shotgun proteomics can delineate the correlation between transcription and translation of gene products, but provide no assessment of protein function or activity. As many enzymes are expressed in zymogen or inhibitor bound proteoforms until post-translational and/or proteolytic activation occurs (4), alternative approaches are needed to measure protein activity. ABPP is a powerful chemical biology approach that specifically targets and provides a direct readout of functionally active enzymes within the proteome, facilitating analysis of functional activity. By targeting an ABP-reactive subset, ABPP reduces proteome complexity facilitating measurement of low abundance proteins that are otherwise undetectable (4). We endeavored to compare ABP-reactive proteins between three aspergilli: the pathogenic Af, and the closely related but rarely pathogenic Nf and Ac in an attempt to identify proteins that may contribute to pathogenicity.

A direct evaluation and comparison of ABP-reactive proteins within Af and closely related aspergilli can provide new insights into the ability of Af to cause the devastating lung disease IA. Approximately, 8% of the Af genome is organism-specific; most of these genes are located in subtelomeric regions, but appear to have limited contribution to initiation of infection (10). Virulence associated genes of Af have detected orthologs in Nf and Ac, as well as more distantly related fungi such as S. cerevisiae and Cryptococcus neoformans (10). Previous studies found, a large number of Af proteins with Nf and Ac orthologs were induced during early murine model infection (72). Because known virulence associated genes are present in nonpathogenic aspergilli, we hypothesize that differential protein regulation induced by external stimuli plays an important role in distinguishing between the pathogenicity of Af, Nf, and Ac. Therefore, an ABPP comparative analysis of the three species greatly complements knowledge of virulence in aspergilli, and identifies reactive proteins as candidate markers for clinical detection of human infection. Af can adapt to and grow in the presence of human serum (28), and during infection Af can disseminate to other organs from the lungs via blood vessels (73). We identified ABP-reactive proteins of Nf and Ac grown in the presence of HS, and compared ABP-reactivity among all three closely related aspergilli.

Aspergilli are saprophytic filamentous fungi that are able to adapt well to environmental stressors such as nutrient limitation. The saprophytic lifestyle allows these fungi to obtain enough nutrients for growth from diverse sources. As expected, all three fungi grew well in the presence of 10% HS (Fig. 1). Af produced the maximum biomass per ml in the presence of HS, and all three fungi reached maximum biomass by 48 h of growth. Growth rates of multiple isolates of Af have been correlated with pathogenicity (74), and we speculate the higher biomass production of Af in the presence of HS may be associated with opportunistic infection.

Abpp Detected Proteins Unique to Aspergillus fumigatus—We detected only 50% of Af orthologous proteins in Nf and Ac regardless of culture condition or duration. As previously shown, detection of probe-reactive proteins is independent of global protein abundance (5), and therefore protein measurement is dependent on protein–probe interaction. Detailed analysis of reactive proteins showed that specific proteins associated with virulence and stress response were measured in Af, but absent in Nf and Ac analyses. In fact, ≤23% of virulence associated protein orthologs were observed in Nf and Ac. However, the identification of virulence-associated proteins in Af was not exclusive to HS culture, reiterating their apparent role in primary biological processes such as growth, de novo biosynthesis, and cell wall integrity. Additionally, glutathione biosynthesis and hydrogen peroxide catabolism, both associated with oxidative stress, were absent in Nf and Ac samples. Most other biological processes and essential genes were observed in all three organisms and culture conditions. Six proteins associated with response to oxidative and chemical stress were exclusive to Af with HS culture at 48 h, and completely absent in Nf and Ac. A robust ABP-protein reactivity response to host induced stress may contribute to initiation and maintenance of IA. The remaining unique Af proteins were observed in both AMM and HS culture and belonged to Biological Process GO slim categories shared with Nf and Ac. Although Biological Process GO terms and slim categories were consistent between the three organisms, the number of GO slim occurrences, and the number of proteins associated with GO terms for Af was almost twofold greater than Nf or Ac.

Comparison of Protein Reactivity—ABPP facilitates analysis of ABP-protein reactivity patterns between conditions; based on the similar shape growth curves in the presence of HS for each fungus, we expected to see similar trends in ABP-protein reactivity over time. In Af and Nf, the number of ABP-reactive proteins increased over time in the presence of HS (24 versus 48 h HS, Fig. 6). However, in Ac many more ABP-reactive proteins were measured at 24 h culture in both culture conditions. It appears that the Af and Nf response to HS is hallmarkd by increasing ABP-protein reactivity over time, whereas Ac response to HS is fundamentally different. Particularly for Nf, the induction of ABP-protein reactivity over time in HS is a direct result of the presence of HS and not growth phase because at stationary phase in AMM and HS culture, many more ABP-reactive proteins are measured in HS culture than AMM culture (Fig. 6). If growth phase dictated ABP-protein reaction, we would expect the same number of measured proteins over time with and without HS. Therefore, HS induces the number and reactivity of proteins that interact with the ABPs independent of microbe growth phase, and is anticipated to be applicable to Af cultures with HS if ABPP was performed at stationary phase. The induction of ABP-protein reactivity in HS over time may have mechanistic implications for Af’s ability to cause IA.
ABPP of Pathogenic and Nonpathogenic Fungi

![Diagram of Fungal Morphology](image)

Function Induced by Human Serum—Another factor associated with opportunistic infection is response to environment (73). According to the growth curves (Fig. 1), all three aspergilli respond to HS by inducing early log phase growth in comparison to AMM alone; however, only in Af has HS induced observation of ABP-reactive proteins associated with growth. To specifically analyze function induced in HS culture, we compared the proteins more reactive in Af, Nf, and Ac during 24 h HS culture compared with AMM culture. The biological processes associated with high ABP-protein reactivity in 24 h HS culture of Af appear to be associated with robust growth of the fungus (Figs. 5). In Af, many proteins involved in cell, organelle and cytoskeletal biogenesis, cell wall and cell membrane biosynthesis, fatty acid and lipid metabolism, and protein transport have induced ABP-reactivity in HS, yet in Nf and Ac fewer proteins in these pathways have higher ABP-reactivity in HS culture implying poorer response to HS. These pathways have been linked to each other in S. cerevisiae via ergosterol and lipid signaling (75).

ABPP Identified Signaling Pathway Proteins Regulated Over Time—Signaling pathways are essential to fungal morphology because they facilitate appropriate organism response to environmental cues. Aspergilli are particularly adept at adapting to different environmental stressors because of their saprophytic lifestyle. We observed protein receptors and regulating proteins that participate in signaling pathways (Table IV, Fig. 8). The Rho GTPase, MpkA, Rac GTPase, and RasA GTPase have roles in cell wall signaling and response to oxidative stress (46, 70, 71, 76). The RasA GTPase is required for proper polarization via cdc42 and MAP kinases, and both RasA and Rac regulate vegetative growth, and asexual development (68, 70). In fact, too much or too little RasA regulation negatively affects growth (77). Furthermore, GpaA stimulates hyphal extension but inhibits asexual development (67). The induction of these, sometimes conflicting, related signaling proteins over time suggests that Af is responding to stress and signals such as nutrient depletion. The reason for induction of ABP-reactive proteins attributable to vegetative growth during stationary phase, when no accumulation of biomass was actually measured, is unclear.

In Af under both culture conditions, signaling protein ABP-reactivity is generally higher at the later time point. However, in Nf, the observed orthologs have higher ABP-reactivity in HS culture over time, but lower ABP-reactivity in AMM culture over time. Furthermore, in Ac all of the observed orthologs have lower ABP-reactivity over time in both culture conditions. This suggests that in both Af and Nf vegetative growth, cell polarity, and conidiation are being regulated during stationary phase in 10% HS. The most likely cause for activation of these signaling pathways over time is nutrient limitation after 48 h of growth (78, 79). Most intriguing is the direct comparison of Nf signaling protein activity at 48 h with and without HS present. The Rac, MpkA, MpkB, GpaA, and SakA all have higher ABP-reactivity in 10% HS culture even though the fungus is in stationary phase at 48 h under both conditions. It is surprising that pathways involved in cell wall integrity and vegetative and polarized growth are active during stationary phase when biomass production is static. Although this response is not unique to Af, reactive signaling pathways because of environmental cues such as human serum may have important implications to successful opportunistic infection.

Clinical Relevance—Our current ABPP study found at least four proteins with HS induced ABP-reactivity that have promise for facilitating detection of Af and possibly to antifungal development. The highly reactive actin cytoskeleton protein Vip1 and FahA, exclusive to HS Af cultures, were not detected in either Nf or Ac. These proteins are antigens of IA patient serum and may be helpful in IA diagnosis (54). The HS induced ABP-reactivity essential 1,3-β glucan Fksp is already an antifungal drug target of caspofungin (66). Finally, the farnesyl-diphosphate farnesyltransferase Erg9, which was exclusive to Af and had HS induced reactivity, could be another target for inhibition of the ergosterol pathway potentially circumventing the incidence of Cyp51a azole resistance (63).

In summary, we have used an ABPP approach to compare the functionally probe-reactive proteome of three closely related aspergilli cultured in the presence of HS. Using two ABPs we identified almost 800, 500, and 400 probe-reactive proteins in Af, Nf, and Ac respectively. 360 reactive proteins, including seven virulence proteins, were unique to Af. Analysis of the reactive proteins showed significant overlap between biological process GO terms for each fungus. However, a closer analysis of the data sets by comparing ABP-reactive protein abundance over time and across condition revealed significant differences between the pathogenic and two non-pathogenic species. Most notable were differences in signaling pathways over time even with consistent growth phase. Furthermore, we showed that Af growth in HS was charac-
ABPP of Pathogenic and Nonpathogenic Fungi

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This article contains supplemental Fig. S1 and Tables S1 to S7.
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