

DATASET BRIEF

A two-dimensional proteome map of the aflatoxigenic fungus *Aspergillus flavus*

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The filamentous fungus *Aspergillus flavus* is an opportunistic soil-borne pathogen that produces aflatoxins, the most potent naturally occurring carcinogenic compounds known. This work represents the first gel-based profiling analysis of *A. flavus* proteome and establishes a 2D proteome map. Using 2DE and MALDI-TOF-MS/MS, we identified 538 mycelial proteins of the aflatoxigenic strain NRRL 3357, the majority of which were functionally annotated as related to various cellular metabolic and biosynthetic processes. Additionally, a few enzymes from the aflatoxin synthesis pathway were also identified.

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Aspergillus flavus is a saprophytic widely distributed pathogen that infects important agricultural commodities worldwide, including maize [1, 2] and oilseed crops such as peanuts, cotton, and tree nuts [3–5]. Being highly carcinogenic, aflatoxins pose a serious health hazard to animals and humans via digestion of contaminated food and feed. Epidemiological studies showed that aflatoxins have been directly linked to the hepatocellular carcinoma [6]. In addition, *A. flavus* is able to cause invasive mycoses in immuno-compromised patients [7].

The field of filamentous fungal proteomics is relatively young, however, a number of studies have been conducted for the *Aspergilli* genus including both profiling and comparative investigations [8–15]. Moreover, proteomic studies of *A. flavus* are now facilitated by the availability of its genomic sequence

[16]. In this paper we present the first proteome profile and 2D proteome map of the whole cell mycelial extract of *A. flavus* wild-type strain NRRL 3357. The description of *A. flavus* proteome grants an important insight into the basic biology of this organism and provides the base for future proteomic investigations.

A. flavus strain NRRL 3357 was obtained from USDA Southern Regional Research Center, New Orleans, LA, USA. To subculture the spores, 10 µL of glycerol stock was spread on potato dextrose agar plates and incubated for 6 days at 28°C. Spores were harvested with 0.5% Triton X-100 solution. Fifty milliliters of potato dextrose broth (PDB) was inoculated to give a spore concentration of 10⁷ conidia/mL. Cultures were incubated in triplicates on rotary shaker for 6 days at 28°C and 150 rpm. Aflatoxin presence in PDB media was assessed via TLC assay as described in Supporting Information paragraph S1.1 and Fig. S1. Mycelia were harvested by filtration through a Buchner funnel, washed with

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Abbreviation: PDB, potato dextrose broth

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double-distilled water, tap-dried, immediately frozen in liquid nitrogen and stored at -80°C . For proteomic studies, 500 mg of frozen mycelia from each triplicate growth was cryogenically ground using GenoGrinder 2000 (SPEX CertiPrep, Metuchen, NJ, USA) to a fine powder.

Proteins were extracted using TCA/acetone precipitation [17] with the following modifications: *A. flavus* mycelial powder (500 mg) was mixed with 5 mL of acetone containing 10% TCA and 0.07% DTT. Proteins were allowed to precipitate for 4 hours at -20°C and collected via centrifugation at $15\,000 \times g$. Precipitated proteins were washed three times with acetone containing 0.07% DTT that had been chilled to 0°C , air-dried and stored at -20°C .

Protein separation, protein visualization, gel scanning, software analysis, and in gel digestion were performed according to procedures that we published previously [18]. In particular, the 2DE was carried out in PROTEAN IEF Cell (Bio-Rad, Hercules, CA, USA) and PROTEAN II XL cell (Bio-Rad), as described in details in Supporting Information paragraph 2. Resolved proteins were visualized with Coomassie Brilliant Blue R-250. Gels were scanned with VersaDoc 3000 imager (Bio-Rad) and analyzed with PDQuest 7.2.0 software (Bio-Rad). Three 2D replicate gels were used for analysis and only spots present in all three replicate gels were selected for identification. The matching summary is shown in Supporting Information Table S1.

Gel spots were excised using Spot Cutter (Bio-Rad), and subjected to automatic in-gel digestion on a ProPrep robotic digester (Genomic Solutions, Ann Arbor, MI, USA). The method included disulfide bond reduction with 10 mM DTT for 10 min at 60°C , alkylation with 100 mM iodoacetamide for 35 min at 25°C , and digestion for 6.5 h at 37°C in 35 μL of 5 ng/ μL trypsin (sequencing grade-modified trypsin; Promega, Madison, WI, USA) and 25 mM ammonium bicarbonate. Resulting tryptic peptides were desalted with C18 Zip-Tips and spotted on ABI 01–192–6-AB MALDI plate (Applied Biosystems, Foster City, CA, USA) in 70% ACN, 0.1% TFA and 5 mg/mL matrix (alpha-cyano-4-hydroxycinnamic acid).

MS analysis and protein identification were performed using the ABI 4700 MALDI TOF/TOF mass spectrometer (Applied Biosystems) and the ABI GPS Explorer V3.6 software, as published previously [19] (see Supporting Information paragraph 3 for details). Briefly, the spectral data from MS scan, 1st and 2nd MS/MS RDA were analyzed together in combined PMF and MS/MS MASCOT search against the *A. flavus* subset of UniProtKB database [20] (as of August 2011). If the proteins were identified as “putative uncharacterized,” the homologous sequences were found using the BLAST search (BLOSSUM62) algorithm against the UniProtKB (August 2012). The following BLAST search criteria were applied: minimal sequence similarity of 40% and E-value cutoff of 1×10^{-20} . Gene ontology (GO) annotations [21] for identified proteins were retrieved from the AgBase database [22] for *A. flavus*.

Approximately 900 protein spots were resolved on large format 2D gels, of which 773 were present in all gel repli-

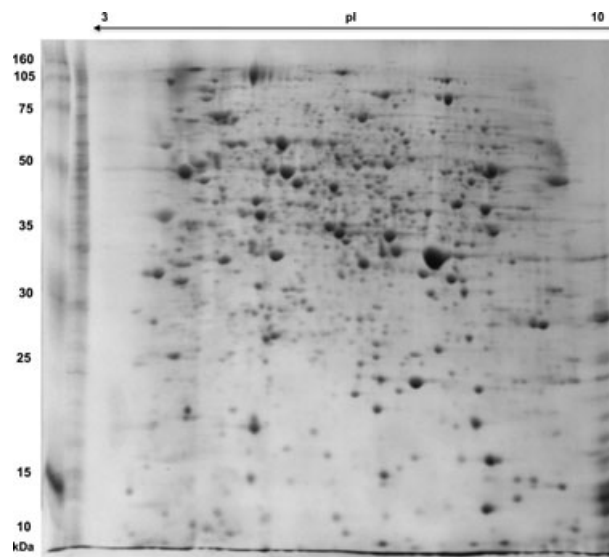


Figure 1. A representative 2D gel of *A. flavus* proteins that were extracted from mycelia grown in PDB media for 6 days. Proteins were separated by IEF on 17-cm IPG strips with nonlinear 3–10 pI gradient followed by SDS-PAGE on large format 12% polyacrylamide gel and visualized with Coomassie Brilliant Blue R-250.

cates. A representative 2D gel is shown in Fig. 1. Reproducible spots were excised and analyzed and 677 of them (87.6%) were identified (Supporting Information Fig. S2 A–C and Table S2). The complete image of the proteome reference map can be viewed at http://lsbi.mafes.msstate.edu/support_data/aspergillus/2-DE_Composite.ppt.

Detailed information pertinent to peptide and protein identification can be found at http://lsbi.mafes.msstate.edu/support_data/aspergillus/Aspergillus_proteome_map.xls. Confident protein identification results and complete mass spectra/identification data set have been also submitted to the Proteomics Identification Database (PRIDE) [23] in two related entries (experiments numbers 20985 and 22722, respectively). Because 133 proteins (24.7%) were present in multiple spots on the 2D gels, 677 identified spots corresponded to 538 distinct protein species. Simultaneously, 88 spots (13%) contained multiple proteins. The molecular masses of identified proteins varied between 5.8 and 213.6 kDa and their isoelectric points ranged from 4.1 to 11.4. The most redundant proteins included translation elongation factor EF-2 subunit, putative (B8NGN7; spots 326, 327, 328, 89, 99, 263, 265, 122) and mycelial catalase Cat1 (B8NLE2; spots 26, 27, 28, 29, 200, 202, 203, 205), both present in eight spots. Some of the most abundant proteins (the strongest spots) comprised glyceraldehyde-3-phosphate dehydrogenase (spot 420), ATP synthase beta subunit (spots 1 and 340), pyruvate decarboxylase PdcA (spot 2), enolase (spot 3), mycelial catalase Cat1 (spot 27), superoxide dismutase (spot 573), and peptidyl-prolyl cis-trans isomerase (spot 618). Thirty-three proteins (6.1%) were identified as putative uncharacterized. Sequences of these proteins were used in BLAST searches

against UniProtKB, and homologous proteins were found for 18 uncharacterized identifications in 10 different fungal species (Supporting Information Table S3). Twelve homologous proteins had sequence similarity above 70% and only three had sequence similarity below 50%. For the 15 uncharacterized proteins, no homologous proteins satisfying BLAST criteria were found.

Functional classification of all identified proteins was performed using GO annotations, and 466 proteins (86.6%) had assigned total of 3759 GO terms for all three aspects of GO: Molecular Function, Biological Process, and Cellular Component.

For Molecular Function, annotations were available for 448 proteins (83.3%) and were represented by 2082 GO terms from 285 diverse functions that were clustered into 13 GO slim categories (Fig. 2A). General category catalytic activity (30.4%) was the most abundant, followed by binding (15.4%), nucleotide binding (15.9%), hydrolase (13.6%), and transferase activities (11.6%). Some functions such as nucleic acid binding (3.8%), kinase activity (2.6%), transporter activity (2.7%), and structural molecule activity (1.8%), were present in smaller numbers. The terms enzyme regulator activity (0.2%), receptor activity (0.3%), and antioxidant (0.7%) activity are examples of under-represented functions.

Annotations for Biological Process were found for 415 (77.1%) mycelial proteins, and 1262 assigned GO terms belonging to 597 cellular processes were merged into 16 GO slim categories (Fig. 2B). Most terms were assigned to the general metabolism category (41.9%). Central metabolic pathways such as metabolism of carbohydrates, lipids, nucleic acids, amino acids, and proteins contributed with 3.2, 0.6, 7.5, 6.8, and 7.4% of GO terms, respectively. Enzymes from glycolysis and tricarboxylic acid cycle dominated the carbohydrate metabolism category and were among the most abundant protein spots on 2D gels. A number of terms belonged to biosynthetic process (14.6%) and it mainly contained proteins from translation (ribosomal proteins, aminoacyl-tRNA synthetases, initiation and elongation factors, etc.) and biosynthesis of vitamins (e.g., biotin, pyridoxine, riboflavin, and folic acid) and cofactors. Approximately 7.3% of terms were allocated to transport, including transport of proteins, small molecules, and ions. This group contained porins, carriers, and numerous subunits of membrane-associated proteins from the respiratory chain. Regulatory proteins (1.4% GO terms) were also identified and included proteins that regulate transcription, translational elongation, and termination as well as actin filament polymerization. Lastly, a subset of terms was designated to Cellular Component organization (2.1%), cellular homeostasis (0.4%), utilization of sulfur, phosphorus and nitrogen (2.2%), signaling (0.2%), and response to stress (0.7%). The latter contained mycelial catalase Cat1 (B8NLE2; annotated as “responsive to oxidative stress”) that was also one of the most abundant and redundant spots on the *A. flavus* 2D map. One hundred twenty-three *A. flavus* proteins did not have assigned GO term for Biological Process and are therefore listed as unclassified. The association of these proteins

with particular cellular processes and/or biochemical pathways will be revealed as more functional data for *A. flavus* will become available in AgBase and other public databases.

Finally, the distribution of *A. flavus* proteins among cellular compartments was also established, however, only 161 proteins (32%) were annotated by 414 Cellular Component GO terms. The identified proteins belonged to 55 cellular compartments and protein complexes, which were further summarized into 13 GO slim categories (Fig. 2C). A high fraction of proteins were assigned general terms cell and intracellular, which accounted for 14.7 and 18.2% terms, respectively. Highly abundant terms were for cytoplasm (13.0%) and protein complexes (11.9%). Mitochondrion, nucleus, ribosome, membrane, and cytoskeleton accounted for 3.1, 4.4, 3.7, 3.4, and 2.1% of GO terms, respectively. From 21 proteins annotated as “membrane” only four had a single transmembrane domain, which was expected since multiple transmembrane domain-containing proteins are not compatible with 2DE. A small fraction of terms (<1%) were localized to the extracellular region (0.3%) and endoplasmic reticulum (0.8%).

For this study, *A. flavus* NRRL 3357 was grown in PDB media, which is a general media routinely used for cultivation of yeasts and fungi. When grown at 28°C, this isolate produced aflatoxins B1 and B2 for prolonged period of time, as shown in Supporting Information Fig. S1. In addition, gene expression studies indicated that aflatoxin biosynthesis peaks at 28°C [24].

We indeed detected the aflatoxin itself (Supporting Information Fig. S1) and identified O-methyltransferase A (omtA; Q9P900; spot 235) and AflK/ vbs/ VERB synthase (B8NHY3; spot 215), two of the key enzymes in aflatoxin synthesis pathway [25, 26]. Other proteins such as ver-1 (Q5VDE3; spot 475), norA (Q5VDE4; spot 402), and aflatoxin B1-aldehyde reductase GliO-like (B8N4U6, spot 416) are also gene candidates possibly related to aflatoxin metabolism.

When compared to the recently published mycelial 2D proteome of *A. fumigatus* [9] more GO categories were associated with *A. flavus* dataset (Biological Process: 597 versus 139; Cellular Component: 55 versus 41). This is obviously due to higher number of proteins identified in our study (538 versus 334 *A. fumigatus* proteins) and better annotations for *A. flavus* (86.6% versus 60% terms assigned). Although grown under different culture conditions, major cellular processes were represented in both fungi, where housekeeping enzymes of primary metabolic processes and diverse biosynthetic pathways were among the most prominent proteins. The major difference was the presence of aflatoxin biosynthetic enzymes in *A. flavus* proteome and their absence in *A. fumigatus* dataset. In regards to Cellular Component, similarities were observed for subcellular distribution of proteins. Both intracellular proteomes were mainly comprised of cytoplasmic proteins and components of protein complexes. One of the notable differences included higher number of ribosomal proteins in *A. fumigatus* proteome (5.2% versus 3.7%) evidently due to the wider pH range used for isoelectric focusing resulting to a better separation of basic ribosomal proteins.

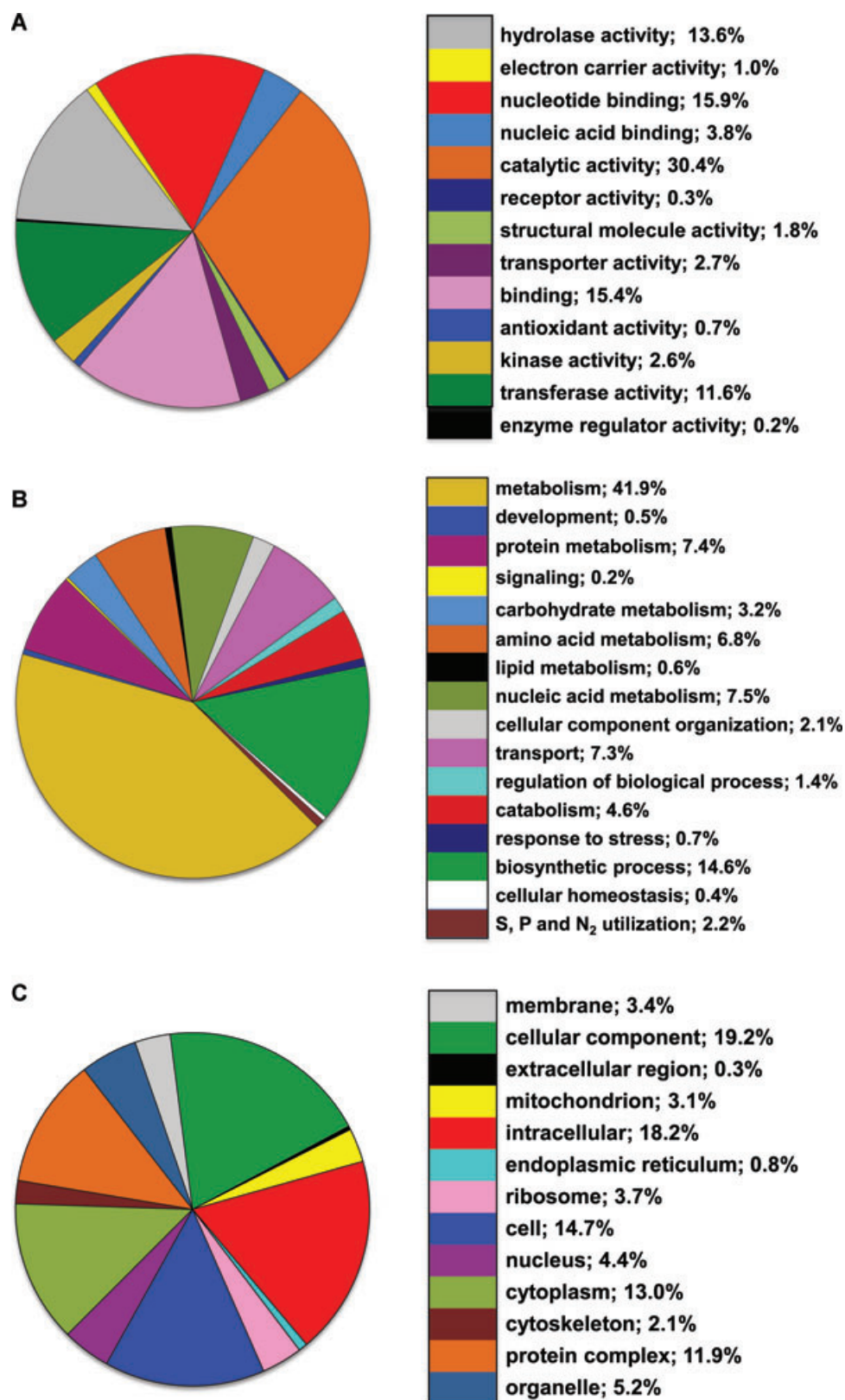


Figure 2. Classification of *A. flavus* mycelial proteins based on gene ontology annotations for (A) Molecular Function, (B) Biological Process, and (C) Cellular Component. Numbers in percentages (%) correspond to the numbers of GO terms assigned for a particular GO category.

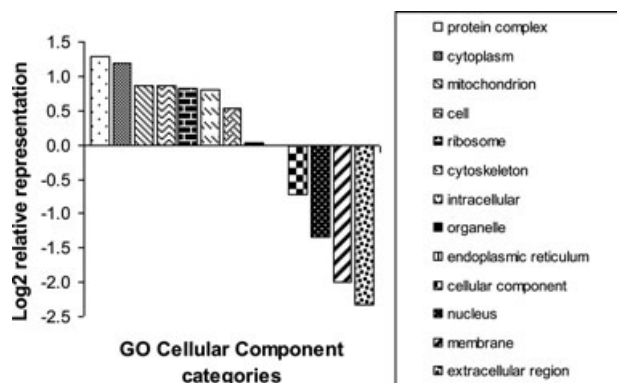


Figure 3. Over- and underrepresentation of GO Cellular Component categories in *A. flavus* proteome. The experimental dataset was compared to the entire *A. flavus* UniProtKB dataset and plotted as $\log_2$ of ratio of percentual representation of GO terms assigned for common GO categories.

Additionally, theoretical annotations were performed for the entire predicted proteome of *A. flavus* (as per UniProtKB database). For Biological Process, 644 GO categories were retrieved, compared to 597 for the presented experimental dataset, which for instance, did not contain proteins related to cell wall metabolism such as cellulose and pectin catabolism—processes commonly associated with extracellular/secreted proteomes. Further absent GO categories consisted of DNA repair, mitosis, tRNA processing, and others. Examples of missing categories for Cellular Component (181 theoretical versus 55 experimental) included cell wall, nucleolus, signal recognition particle, exocyst, and spindle pole. For GO categories common for both experimental and theoretical dataset, the relative representation plot was constructed as shown in Fig. 3. It demonstrates methodology bias towards cytoplasmic and generally well soluble proteins, while the proteins of nucleus and membrane are underrepresented in proteome resolved by 2DE gels. As expected, proteins of the extracellular regions are also poorly represented, as they are predominantly present in growth media [8], while our study concentrated on the intracellular mycelial proteome.

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The authors have declared no conflict of interest.

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