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## The allergenicity of *Aspergillus fumigatus* conidia is influenced by growth temperature

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### ABSTRACT

Common indoor and outdoor environmental fungi such as *Aspergillus fumigatus* produce asexual spores containing a collection of proteins that can bind IgE antibodies and trigger allergic reactions. We characterized the impact of sporulation temperature on the IgE-binding capacity (allergenicity) of *A. fumigatus* and explored the links between variable allergenicity and temperature-dependant expression of genes encoding these allergenic proteins. A 12-fold increase in *A. fumigatus* allergenicity per spore was observed when sporulation temperatures were decreased from 32 °C to 17 °C. Per spore protein mass and Asp f 1 allergen mass also followed this trend. Functional gene expression analysis of *A. fumigatus* sporulating cultures by real-time reverse-transcription PCR and gene expression microarrays revealed that a greater number of genes encoding known, major allergens are more highly expressed at lower sporulation temperatures. The results of this study indicate that environmental conditions at growth significantly influence the allergenicity of this common mould through the differential production of allergenic proteins, and highlight the importance of *in vivo* or *in vitro* allergenicity measurements for understanding environmental exposure to airborne allergenic fungi.

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### Introduction

*Aspergillus fumigatus* is a saprotrophic fungus commonly found in the environment that is also an opportunistic human pathogen and a major airborne allergen (Ronning *et al.* 2005). This fungus produces large quantities of asexual spores (conidia) (Mah & Yu 2006) that are 2–3 µm in diameter, thus allowing inhaled conidia to reach terminal airways and alveoli. The presence of *A. fumigatus* in outdoor and indoor aerosols is common (Latge 1999) and inhalation of *Aspergillus* spores is associated

with a variety of allergic respiratory diseases, including allergic rhinitis, sinusitis, and asthma. More allergenic proteins have been characterized in *A. fumigatus* than in all other fungi combined (Ronning *et al.* 2005). To date, *A. fumigatus* contains 22 known allergenic proteins (IUIS 2008); the spectrum of these includes acid ribosomal proteins, enzymes such as proteases, and heat-shock proteins (Simon-Nobbe *et al.* 2008).

Environmental factors may impact the quantity and allergenic properties of airborne fungi. Long-term field experiments at elevated CO<sub>2</sub> concentrations (Klironomos *et al.* 1997) and

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temperatures (Gange et al. 2007) have indicated the likelihood of increased fungal biomass under current climate change scenarios. In addition, numerous studies on spore development and pathogenicity suggest that environmental conditions during spore development result in physiological changes and differential gene expression in fungal spores (Misaghi et al. 1978; Phillips 1982; Abbas et al. 1995; Bertolini & Tian 1997; Wolf et al. 2010). Since many fungal species including *A. fumigatus* have multiple, unrelated allergenic proteins, environmental factors during sporulation may affect the regulation of genes encoding for these proteins and therefore impact the per spore allergenicity (determined here as the capacity to bind with human IgE from patients sensitized to *A. fumigatus* allergens) (Miguel et al. 1996). Despite the health significance of this potential link between environmental factors and human allergen exposure, the allergenicity of fungal spores produced under different environmental conditions has not been addressed.

Humans are exposed to *A. fumigatus* spores that can potentially be produced under a diversity of indoor and outdoor temperatures. We hypothesize that sporulation temperature will impact the per spore allergenicity of common environmental moulds. To test this hypothesis, human IgE-binding was used to assess the relative allergenicity of *A. fumigatus* spores formed under sporulation temperatures ranging from 17 °C to 32 °C. The origin of variable allergenicity was studied by coupling these analyses with spore protein and Asp f 1 mass (a major allergen of *A. fumigatus*) measurements, and expression profiling of genes that encode for the 22 *A. fumigatus* allergenic proteins.

## Materials and methods

### Preparation of *Aspergillus fumigatus* conidia

The cultivation of *A. fumigatus* (ATCC #34506) took place under three controlled temperatures (17 °C, 25 °C, and 32 °C). At each temperature, fresh cultures of *A. fumigatus* were plated onto potato dextrose agar (PDA) plates and incubated at the respective temperature for 7 d. After the incubation period, the conidia were harvested using flocked, nylon fibre tipped swabs (Copan Diagnostics, Murrietta, CA) and resuspended in 2 ml PBS-T (0.14 M NaCl, 0.01 M phosphate, 0.05 % Tween 20, pH = 7.4). Concentrations of the various conidia suspensions ranged from 10<sup>6</sup> ml<sup>-1</sup> to 10<sup>9</sup> ml<sup>-1</sup> as determined by light microscopy direct counts using a 2 × 10<sup>-5</sup> ml haemocytometer at 400× magnification. To extract protein for the allergenicity inhibition immunoassay, glass beads (0.3 g of 100 µm diameter and 0.1 g of 500 µm diameter) were added to the 2 ml suspensions, and spores were lysed by beadbeating (BioSpec Mini-Beadbeater, Bartlesville, OK) at 2500 rpm for 30 sec. Lysed cells were centrifuged and the protein containing supernatant was collected for further analysis. Protein content was determined using the bicinchoinic acid assay (ThermoFisher Scientific, Waltham, MA) in accordance with the manufacturer's protocol.

### Inhibition immunoassay for allergens

Human serum samples containing allergen-specific IgE from seven patients who demonstrated a clinical sensitivity to

*Aspergillus fumigatus* allergens were obtained from PlasmaLab International (Everett, WA). Estimates of the specific IgE levels of the respective donors using the CAP-RAST (RadioAllergo-Sorbent Test) and/or the ImmuLite 2000 chemiluminescent enzyme immunoassay, as provided by the supplier, are listed in Table S2. The serum samples were pooled and diluted (1:10) in PBS-T before use. For each triplicate experiment at each temperature and spore concentration, 50 µl of the fungal spore protein extract was mixed with an equal volume of the diluted pooled serum mixture in six separate wells of a standard polypropylene 96-well microtiter plate, and incubated at room temperature on a titre plate shaker for 2 h. Allergens in the fungal extracts bind to the IgE in the serum mixture thus decreasing the concentration of free IgE. The spore extract-human serum mixtures were then transferred to MaxiSorp® flat-bottom 96-well plates (Nalge Nunc, Rochester, NY) coated with 46 µg ml<sup>-1</sup> of *A. fumigatus* spore extract and incubated for an additional 2 h at room temperature. At this stage, only free IgE molecules are able to bind to the allergens coated on the microwells. After washing steps were performed, the amount of bound specific IgE in each microwell was quantified by the addition of a secondary antibody (horseradish peroxidase-conjugated goat monoclonal antihuman IgE diluted 1:16 000) and chromogenic substrate (tetramethylbenzidine (TMB)/peroxide substrate colour developer II), followed by colorimetric detection at a wavelength of 450 nm using the SpectraMax 340PC microplate reader (Molecular Devices, Sunnyvale, CA). The relative allergenicities of the spore extracts were defined as follows:

$$\text{relative allergenicity} = \frac{(\text{OD}_{\text{serum}} - \text{OD}_{\text{sample}})}{\text{OD}_{\text{serum}}} \times 1000 \quad (1)$$

where OD<sub>serum</sub> is the optical density measured for the human serum mixture preincubated with no spore samples, and OD<sub>sample</sub> is the optical density measured for the human serum-fungal spore sample mixture. The allergenicity values thus obtained were then averaged across the replicates to obtain the allergenicity measure and associated standard error values for the spores produced at each study temperature and concentration. The final allergenicity per spore value was then determined by plotting allergenicity versus spore concentration for each temperature and estimating the allergenicity per spore and standard error from the slope of the linear portion of this dose-response relationship (Fig S1). Cross reactivity controls comparing the allergenicity of *A. fumigatus* to that of the nontarget *Cladosporium cladosporioides* spores demonstrated that at 10<sup>8</sup> spores, *A. fumigatus* allergenicity was ~10 times greater than that measured with *C. cladosporioides* spores and that the *C. cladosporioides* allergenicity was not statistically different than serum only controls (*t*-test, *p* < 0.05).

### Plate counts for spore samples

To determine the culturable fraction, spores were cultivated at 14 °C, 17 °C, 20 °C, 24 °C, 28 °C, and 32 °C, harvested, and serial 10-fold dilutions containing a known amount (by direct count) of these spores plated onto PDA and incubated at room temperature as is commonly done for the enumeration of environmental fungi. After an incubation period of 8 d, the number of colony forming units (CFU) were counted and

expressed as a percentage of the initial number of spores plated. The percentages at each temperature were averaged across three experimental replicates to obtain the culturability of spores for each study temperature.

### Sporulation gene expression study

To investigate the relationship between allergenicity and expression of genes encoding *Aspergillus fumigatus* allergenic proteins, the transcriptome responses of cultures sporulating at 17 °C and 32 °C were compared. The growth regime was based upon previous studies which have investigated and found changes in conidial properties due to transcriptional level changes during sporulation (Parta et al. 1994; Tsai et al. 1999; Sakamoto et al. 2008). Potato dextrose liquid medium was inoculated with  $1 \times 10^6$  conidia  $\text{ml}^{-1}$  and grown in a shaking (200 rpm) culture at 37 °C overnight. At the end of the overnight incubation, the culture was filtered under vacuum, and the harvested mycelia divided and plated onto PDA. One set of plates was incubated at 17 °C, and the other at 32 °C, for 48 h. At the end of the 2-d incubation, the cultures were harvested with a sterile, moistened cotton swab, resuspended in sterile water, and collected under vacuum filtration. Light microscopy verified the presence of conidiophores and conidial chains in both the 17 °C and 32 °C cultures 48 h after induction. The extracted culture was ground under liquid nitrogen using a mortar and pestle, and total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. Contaminating genomic DNA was removed from RNA samples by treatment with 25  $\mu\text{l}$  of 10 U  $\mu\text{l}^{-1}$  DNase I for 45 min at room temperature. RNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm with a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). The purity and integrity of the RNA was checked using  $A_{260/280}$  and  $A_{260/230}$  ratios from the spectrophotometric readings and by denaturing gel electrophoresis.

Microarray glass slides of *A. fumigatus* spotted with 10 003 oligonucleotides (70-mers with duplicate printings each) representing all predicted open reading frames were obtained from the J. Craig Venter Institute (Rockville, MD). Amino-allyl-labelled first-strand cDNA synthesis and indirect Cy dye incorporation of purified RNA from 17 °C and 32 °C sporulating cultures were performed using the standard TIGR (The Institute of Genome Research) protocol (Sheppard et al. 2005), except that a 3:1 aminoallyl-dUTP to dTTP ratio was used. The resulting dye-coupled cDNA was hybridized onto the *A. fumigatus* glass slide microarray using a BioMicro (Salt Lake City, UT) MicroArray User Interface (MAUI) hybridization station at the W.M. Keck Microarray Resource Facility at Yale University. A total of three replicate sporulation experiments were performed, with the 17 °C and 32 °C sporulating culture RNA from each experiment hybridized together onto two separate dye-flip slides to remove dye coupling bias, for a total of six microarray hybridizations. The six microarray slides were scanned using an Axon GenePix 4200 laser confocal scanner (Molecular Devices, Sunnyvale, CA). The generated 16-bit .tiff images were analyzed by TIGR Spotfinder (version 3.1.2) for raw intensity values, which were subsequently imported into TIGR MIDAS (Microarray Data Analysis

System) (version 2.20). Low intensity values were removed with the low intensity filter, and data normalization was carried out using the LOcally WEighted Scatterplot Smoothing (LOWESS) algorithm. Standard deviation regularization was also performed to address print-tip bias. The resulting normalized data was analyzed on Microsoft Excel and genes having significantly differential expression levels between 17 °C and 32 °C sporulating cultures (i.e. more highly expressed at one study temperature relative to the other) were identified using the Wilcoxon–Mann–Whitney test ( $p < 0.05$ ). The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE16617.

To validate microarray data, real-time reverse-transcription PCR (rRT-PCR) was used to measure relative transcript levels of nine genes (including five that encode the known allergens Asp f 1, Asp f 3, Asp f 10, Asp f 15, and Asp f 17) (Table S3) (Breakspear & Momany 2007). Approximately 2  $\mu\text{g}$  of purified RNA from each of the three separate sporulation experiments described above was used in first-strand cDNA synthesis using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The primers used for each selected gene are shown in Table S3, and were either taken from previous studies (Sheppard et al. 2005; Gravelat et al. 2008), or were designed with the assistance of online primer design software (Genscript, Piscataway, New Jersey).

Gene expression was quantified using the FastStart Universal SYBR Green Master Mix (Roche Applied Science, Indianapolis, IN) and an Applied Biosystems Fast Real-Time PCR 7500 system (Applied Biosystems, Foster City, CA). The thermocycling conditions included an initial denaturation for 10 min at 95 °C, followed by 40 cycles of 15-sec denaturation at 95 °C and 1 min for annealing and extension at 60 °C. Three replicates were carried out for each reaction, and negative no-RT-controls were performed on the source RNA to verify the absence of genomic DNA contamination. The endogenous reference gene 18S rRNA was used to normalize the quantity of RNA used, and the relative transcript levels between the two sporulating cultures were determined using the  $2^{-\Delta\Delta\text{CT}}$  method (Livak & Schmittgen 2001).

### ELISA assay for Asp f 1 content in fungal spore samples

An Asp f 1-specific ELISA kit from Indoor Biotechnologies (Charlottesville, VA) was used to measure the content of Asp f 1 in spore samples cultivated at 17 °C and 32 °C. Anti-Asp f 1 mAb 4A6 was used as the capture antibody and polyclonal rabbit anti-Asp f 1 antibody as the detection antibody. Experimental procedures followed manufacturer's recommended protocol, and purified natural Asp f 1 ( $0.04 \text{ ng ml}^{-1}$ – $40 \text{ ng ml}^{-1}$ ) was used to form a standard curve. ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)) substrate (Vector Laboratories, Burlingame, CA) was used for colour development, with colorimetric detection at 405 nm using a SpectraMax 430PC microplate reader (Molecular Devices, Sunnyvale, CA). Optical density values were averaged among replicates for each spore sample and normalized by the respective spore counts ( $\times 10^7$  spores  $\text{ml}^{-1}$ ). Concentrations were then averaged across three biological replicates to obtain

the mean Asp f 1 per spore measure for spore samples produced at each of the two study temperatures.

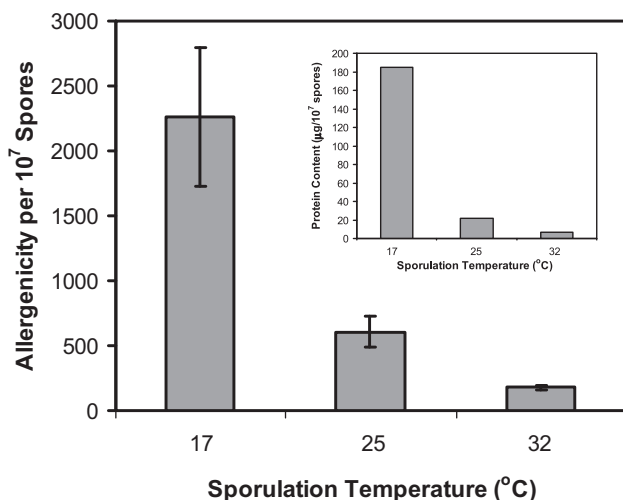
## Results

### Allergenicity per spore increases with decreasing sporulation temperature

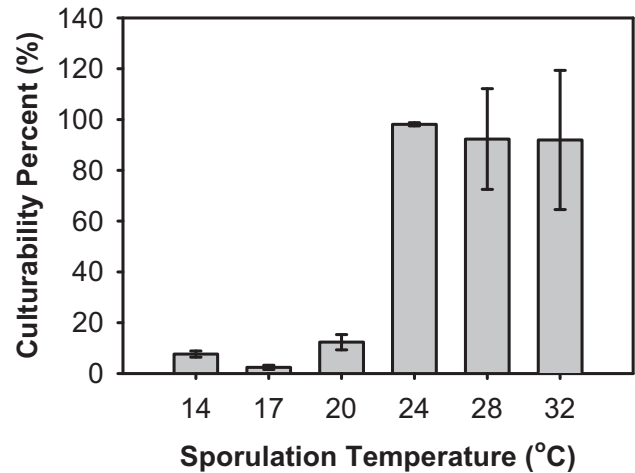
Inhibition immunoassays using serum from patients who demonstrated a clinical sensitivity to *Aspergillus fumigatus* allergens were used to study the effect of different sporulation temperatures on the allergen content of cultivated spores. Assay responses were estimated at each temperature from the linear portion of dose–response curves (Fig S1). Spores were significantly more allergenic on a per spore basis as sporulation temperatures decreased ( $p < 0.05$ , ANOVA) (Fig 1). The allergenicity of spores cultivated at 17 °C was found to be approximately 12-fold higher than conidia that sporulated at 32 °C. Fig 1 Inset demonstrates that protein concentration in spores also exhibited the same trend as allergenicity with a 10 times decrease in protein mass per spore as sporulation temperatures increased from 17 °C to 32 °C.

### Spore culturability increases with increasing growth temperature

Culture-based methods have commonly been used to measure the concentration and distribution of environmental fungi (Burge 2002). To provide a comparison with relative allergenicity measurements in this study, culturable counts were carried out for spores formed at temperatures ranging from 14 °C to 32 °C. These spore culturability results are presented in Fig 2. Culturability was significantly higher ( $p < 0.05$ , t-test) for conidia that had sporulated at 24 °C, 28 °C, and 32 °C, as compared to conidia that had sporulated at 14 °C, 17 °C, and 20 °C. More than 90 % of plated conidia



**Fig 1 – Allergenicity per 10<sup>7</sup> spores of *Aspergillus fumigatus* conidia cultivated under different sporulation temperatures. Error bars represent standard error values over three independent experimental replicates. (Inset) Protein content per 10<sup>7</sup> spores in conidia produced at 17 °C, 25 °C, and 32 °C.**



**Fig 2 – Culturability of *A. fumigatus* spores grown under different conidiation temperatures (± standard error, n = 3 experiments).**

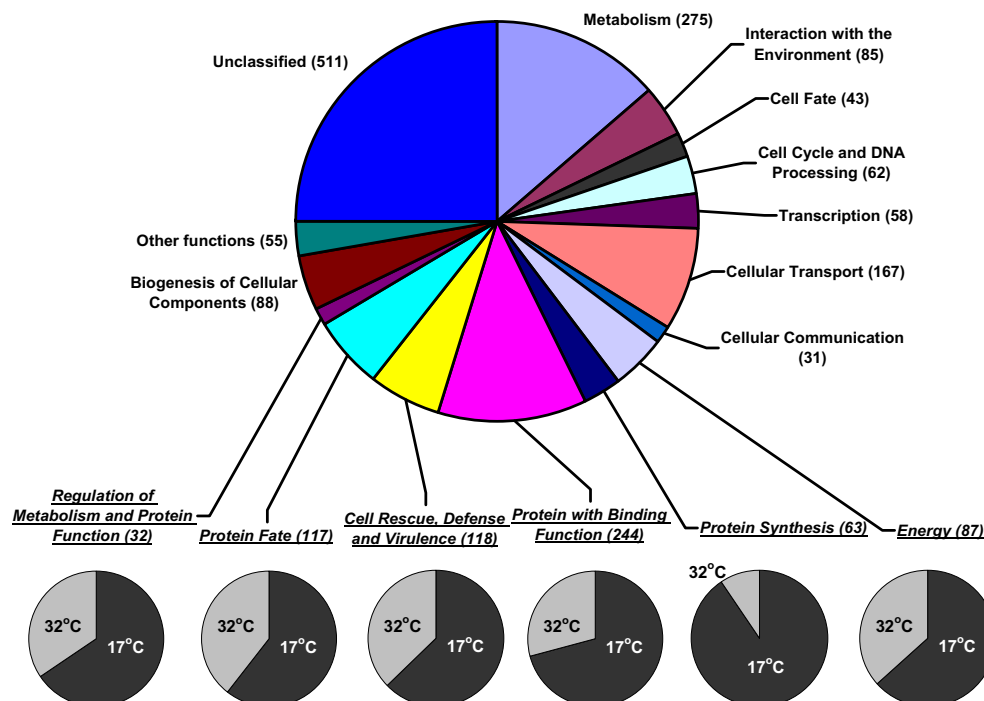
produced at temperatures of 24 °C or higher had developed into CFU after 8 d of incubation. On the other hand, for conidia that had sporulated at temperatures of 20 °C or lower, the percentage of spores that had developed into CFU was ~ 12 % or less. This trend of increasing culturability with increasing temperature is opposite to that observed between allergenicity and sporulation temperature.

### Transcriptome responses under different sporulation temperatures

To investigate whether results from the temperature–allergenicity study could be associated with differing expression levels of genes that encode the variety of known allergens in *Aspergillus fumigatus*, microarray analysis of RNA isolated from sporulating cultures at 17 °C and 32 °C was carried out. A total of 1056 genes were found to be significantly differentially expressed between 17 °C and 32 °C sporulating cultures (Wilcoxon–Mann–Whitney test,  $p < 0.05$ ). Of these, 501 were more highly expressed under sporulation at 17 °C, while 555 were more highly expressed under sporulation at 32 °C. Differentially-expressed genes were classified into functional groups using their assigned Munich Information Center for Protein Sequences (MIPS) functional catalogue (FunCat) annotation in the Protein Extraction, Description and Analysis Tool (PEDANT) for *A. fumigatus* (Frishman & Mewes 1997). Fig 3 shows the number of genes in each functional class that were differentially expressed between the two sporulating cultures.

The total numbers of genes upregulated in one culture relative to the other were not markedly different from each other, suggesting that global transcriptome responses were not repressed by higher or lower sporulation temperatures. Functional classes where genes more highly expressed at 17 °C relative to 32 °C make up the majority (at least 60 %) of the differentially-expressed candidates include the following: energy metabolism (55 of 87 genes); protein synthesis (55 of 61 genes); protein fate (71 of 117 genes); proteins with binding function (171 of 242 genes); regulation of metabolism and protein function (21 of 32 genes); and cell rescue, defence and





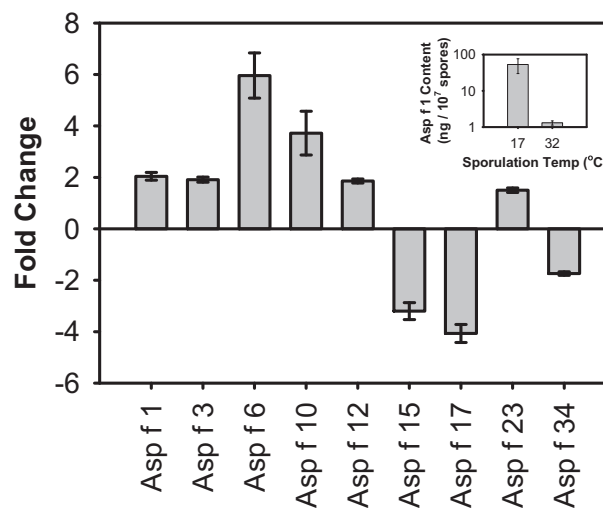
**Fig 3 – Functional classifications of genes with statistically significant differential expression levels between 17 °C and 32 °C sporulating cultures. Numbers in parenthesis represent the total number of differentially-expressed genes within each functional class. In the underlined functional classes and smaller pie charts ■ represents genes more highly expressed at 17 °C and □ represents genes more highly expressed at 32 °C.**

virulence (74 of 118 genes). Genes more highly expressed at 32 °C relative to 17 °C did not form at least a 60 % majority in any of the other major functional classes.

#### Differentially-expressed allergenic genes under different sporulation temperatures

Among the 22 known allergens of *Aspergillus fumigatus*, genes encoding six of these allergens (the major allergens Asp f 1 and Asp f 3, along with Asp f 6, Asp f 10, Asp f 12, and Asp f 23), were found to have significantly higher mRNA levels in 17 °C sporulating cultures versus 32 °C sporulating cultures, while the three genes encoding Asp f 15, Asp f 17, and Asp f 34 were found to have significantly higher transcript levels in cultures that sporulated under the higher temperature (Wilcoxon–Mann–Whitney test,  $p < 0.05$ ) (Fig 4). No significant difference in transcript level was observed for the other 13-allergen encoding genes. The full list of all 22 genes that encode known allergens of *A. fumigatus* is provided in Supplemental Data, Table S1.

To verify the results from microarray analyses, nine genes that were found to be differentially regulated under different sporulation temperatures were selected for relative transcript level determination by rRT-PCR. These genes were chosen because of the focus of the study on allergenic genes, and because they span a wide range of functional classes (e.g. metabolism, protein fate, cell rescue, defence and virulence) and microarray fold change magnitudes (~1.5–7). The results of the rRT-PCR analysis were consistent with the microarray



**Fig 4 – Allergenic gene expression and Asp f 1 protein production in 17 °C and 32 °C spores. Allergen nomenclature follows the official list of the International Union of Immunological Societies. A positive fold change ( $\pm$  standard error,  $n = 3$  experiments) indicates higher expression of the gene in 17 °C sporulating cultures relative to 32 °C cultures, while a negative fold change indicates higher expression in 32 °C cultures relative to 17 °C. (Inset) Asp f 1 content per 10<sup>7</sup> spores ( $\pm$  standard error,  $n = 3$  experiments) in spore samples grown under 17 °C and 32 °C.**

**Table 1 – Relative mRNA levels ( $\pm$  standard error,  $n = 3$  experiments) of nine selected genes. A positive fold change corresponds to higher expression in the 17 °C culture versus the 32 °C culture.**

Accession Number	Target name	Microarray fold change	rRT-PCR fold change
Afu5g02330	Asp f 1 (ribotoxin)	2.04 $\pm$ 0.15	4.41 $\pm$ 0.96
Afu6g02280	Asp f 3 (peroxysomal protein)	1.91 $\pm$ 0.10	4.42 $\pm$ 0.35
Afu5g13300	Asp f 10 (aspartic endopeptidase Pep1)	3.72 $\pm$ 0.85	4.97 $\pm$ 1.41
Afu2g12630	Asp f 15 (allergenic cerato-platanin)	-3.20 $\pm$ 0.33	-19.9 $\pm$ 8.5
Afu4g03240	Asp f 17 (cell wall galactomannoprotein Mp1)	-4.07 $\pm$ 0.35	-48.7 $\pm$ 21.6
Afu6g00430	IgE-binding protein	-3.96 $\pm$ 0.21	-5.82 $\pm$ 2.41
Afu5g06240	Alcohol dehydrogenase, putative	6.84 $\pm$ 1.11	11.3 $\pm$ 2.5
Afu1g07440	Molecular chaperone Hsp70	1.64 $\pm$ 0.09	2.50 $\pm$ 0.87
Afu1g09670	HLH transcription factor, putative	-1.99 $\pm$ 0.07	-3.47 $\pm$ 1.01
Afu1g17250	Conidial hydrophobin RodB	2.19 $\pm$ 0.30	8.10 $\pm$ 3.55

data, with all nine genes tested having expression profiles similar to those observed with the microarrays. Table 1 compares the relative fold changes of the nine tested genes between the rRT-PCR and microarray analyses.

### Asp f 1 content of 17 °C and 32 °C spores

Quantitative ELISA analysis of the Asp f 1 major allergen was performed to determine if protein levels corresponded to transcriptomic results from the microarray and rRT-PCR experiments. The Asp f 1 protein content in spores cultivated at 17 °C and 32 °C are shown in Fig 4 Inset. Asp f 1 levels in 17 °C spore cultures were found to be  $\sim$  40-fold higher than corresponding levels in 32 °C spores, indicating that specific protein production levels were consistent with the greater Asp f 1 gene transcription at 17 °C relative to 32 °C found from both microarray and rRT-PCR analyses ( $\sim$ 2–4.5-fold higher mRNA levels after 48 h of sporulation). This general relationship between the Asp f 1 major allergen and temperature is also in agreement with the total allergenicity and protein content results presented in Fig 1.

## Discussion

### Implications for assessing exposure to environmental allergens

The use of atopic serum containing allergen-specific IgE from patients that demonstrated a clinical sensitivity to *Aspergillus fumigatus* provides a way to quantify the allergenicity of conidia that have sporulated under different temperatures. Allergenicity and protein content per spore increased with a decrease in sporulation temperature, with more than a 10-fold difference in allergenicity between spores produced at 17 °C compared to their counterparts grown at 32 °C. ELISA-based quantification of the Asp f 1 protein, a ribotoxin and major allergen of *A. fumigatus*, has also demonstrated that this allergen is present in significantly greater amounts in spores that sporulated at 17 °C, compared to spores that were produced at 32 °C.

Measuring the IgE-binding capacity allows for an accounting of all known *A. fumigatus* allergens and represents a significant increase in information over traditional methods of environmental allergen assessment that include whole cell

enumeration or measurement of only the Asp f 1 protein. Although IgE-binding capacity may not be a complete representation of the complex *in vivo* response to allergens, previous studies do suggest a relationship between the two. A comparison of IgE-binding with *in vivo* skin test end point titrations for *A. fumigatus* has demonstrated correlations between IgE-binding capacity and the percentage of subjects showing positive skin reactions to a particular *A. fumigatus* extract (Turner et al. 1980). Through more indepth comparative studies conducted for ragweed pollen, a strong correlation between IgE-binding and *in vivo* responses has been observed over a large (1000 fold) range in extract allergenicities (Gleich et al. 1973).

Plate count experiments with spores produced at variable temperatures revealed that the culturability is higher for spores produced above a threshold temperature of 20 °C. This finding is in general agreement with related environmental growth temperature studies. Spores of various fungal species in indoor environments having higher ambient temperatures ( $\sim$ 22 °C) than outdoors ( $\sim$ 11 °C) have demonstrated higher median culturabilities (55 % versus 25 % for all fungi; 9 % versus 2 % for *Aspergillus* spp.), as do outdoor spores sampled during fall ( $\sim$ 37 %) compared to winter ( $\sim$ 8.8 %) (Lee et al. 2006).

These observed temperature–allergenicity and temperature–culturability relationships are significant for assessing exposure to allergens in the indoor environment. Although common practice, estimating exposure by culture-based methods (Burge 2002) has not yielded a strong exposure–response relationship with respiratory symptoms and allergies (Pasanen 2001). The results from this study suggest how these traditional whole cell measurements of allergenic fungi are limited indicators of human health impact. *Aspergillus fumigatus* spores produced at lower temperatures, while less culturable, are shown to be significantly more allergenic on a per spore basis. Thus, culturability assays may severely underestimate the allergenicity of a sample. Though measuring protein content per spore has yielded similar trends with temperature as allergenicity, total protein mass mostly includes nonallergenic proteins that do not provoke an IgE response, and therefore protein content may not be a consistent surrogate for allergenicity. Counting total spores by microscopy or rRT-PCR, while more accurate than culture-based methods, still does not account for the variable allergenicity of a fungal

spore. Fungi can sporulate at different temperatures in response to different seasons or different locations (e.g. in an air-conditioned or heated indoor environment or outdoors) and both indoor and outdoor sources contribute to the spore content of indoor air. For *A. fumigatus*, temperatures between 17 °C and 25 °C could potentially be significant in terms of producing the greatest fold changes in allergenicity and culturability of spores. As this falls within the range of most indoor environments and nonwintertime ambient outdoor temperatures in temperate climates, these environments could have profound implications with respect to producing sharp changes in spore viability and allergenicity.

### Temperature-dependent expression of genes encoding *Aspergillus fumigatus* allergens

The microarray and rRT-PCR analyses demonstrate that a greater number of genes that encode known and major allergens are more highly expressed during sporulation at lower temperatures, thus suggesting a transcriptional component to the temperature–allergenicity relationship observed from the immunoassay experiments. Microarray results revealed that 9 of the 22 genes that encode known *A. fumigatus* allergens were found to be significantly differentially expressed between sporulating cultures at the two study temperatures. Twice as many allergen encoding genes were found to have higher transcript levels at 17 °C relative to 32 °C. Included among the six official allergens that were more highly expressed at the lower sporulation temperature are the two known major allergens, Asp f 1, which has been shown to cause IgE antibody responses in 85 % of patients with IgE antibodies to *A. fumigatus* (Arruda *et al.* 1992), and Asp f 3, a peroxisomal membrane protein present on spore surfaces (Asif *et al.* 2006), which has been shown to elicit 94 % IgE reactivity and whose immunogenic properties have been proven *in vivo* (Simon-Nobbe *et al.* 2008). Major allergens are capable of eliciting a strong response, either because they are present in high quantities, or because they possess strong IgE-binding epitopes (Miguel *et al.* 1999). Asp f 1 protein measurement results further support the hypothesis that higher Asp f 1 gene expressions correspond with higher allergen content in spores.

On a genome-wide scale, a markedly greater proportion of differentially-expressed genes was observed as having higher expression levels at 17 °C (relative to 32 °C) in functional classes that include proteins with binding functions, protein fate, protein synthesis, and regulation of metabolism/protein function. This is consistent with the observation of higher protein content in spores cultivated at lower temperatures (Fig 4 Inset). These protein-based functions suggest that enzymatic activity is increased at lower spore production temperatures. Widespread enzyme activity has also been previously observed in *Alternaria helianthi* under lower conidia production temperatures (Abbas *et al.* 1995). This higher enzymatic activity at 17 °C could be in response to the thermotolerant *A. fumigatus* sporulating in a harsher ambient environment that is markedly different from its 37 °C optimum growth temperature. The greater number of cell rescue, defence and virulence genes found to have significantly higher transcript levels in 17 °C sporulating cultures supports this speculation. While

previous microarray studies have analyzed gene expression changes of *A. fumigatus* under time-course temperature shift (heat shock) conditions (Nierman *et al.* 2005; Do *et al.* 2009), to our knowledge, this is the first investigation into the transcriptional response of the fungus under two separate, independent temperature treatments.

The microarray analysis also revealed active transcription of genes linked with the sporulation process. Transcript levels of the gene encoding conidial hydrophobin RodB (Afu1g17250) were found in both sporulating cultures. RodB is a cell wall structural protein involved in the building of the conidial outer cell wall (Gautam *et al.* 2008), and its transcription verifies the expression of spore-associated proteins during sporulation. Other genes encoding spore-associated proteins found to be actively transcribed in both sporulating cultures include the conidial pigment biosynthesis genes *arp1*, *arp2*, *abr1*, and *ayg1* (Tsai *et al.* 1999). In addition, transcript levels in both sporulating cultures were observed for various genes encoding proteins required for the conidiation process (Lamarre *et al.* 2008), including trehalose synthesis (e.g. Afu3g12100), osmoregulation, (e.g. Afu8g04090) and the conidiphore development protein FbD (Afu1g03210). The transcription of these aforementioned genes confirms light-microscopy results that sporulation was actively occurring in the cultures 48 h after induction, and validates the microarray experimental setup. As RNA for the transcriptional study was extracted from actively sporulating cultures at both temperatures, the difference in eventual culturabilities of the spores produced (Fig 2) is not expected to affect the gene transcription being studied during the sporulation stage.

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## Conclusions

This study has demonstrated that spores of the ubiquitous fungus *Aspergillus fumigatus* become more allergenic as sporulation temperatures decrease. Microarray and rRT-PCR results on known allergen encoding genes as well as total and allergen-specific protein measurements further support a transcriptional basis for this variable allergenicity. Growth and sporulation temperature may significantly affect environmental allergen exposure given that multiple allergenic proteins exist for important allergenic fungi such as *Cladosporium herbarum*, *Alternaria alternata*, and *Penicillium* spp. (Bertolini & Tian 1997) and that exposure occurs at multiple temperatures in indoor/outdoor environments, seasons, and climates. This study establishes a link between sporulation temperature and allergenicity for an important allergenic fungus. Because allergenicity can also be potentially related to sporulation conditions in other fungi, further elucidating the relationship between IgE-binding, transcriptional regulation and *in vivo* responses will improve the ability to draw relationships between the environment and allergenic respiratory diseases.

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## Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.funbio.2011.03.006.

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