Heterogeneity in Pathogenicity-related Properties and Stress Tolerance in *Aspergillus fumigatus* Clinical Isolates

**Daisuke Hagiwara**\(^1\), **Hiroki Takahashi**\(^{1,3}\), **Hiroshi Takagi**\(^4\), **Akira Watanabe**\(^1\) and **Katsuhiko Kamei**\(^1\)

\(^1\) Medical Mycology Research Center, Chiba University
\(^2\) Faculty of Life and Environmental Sciences, University of Tsukuba
\(^3\) Molecular Chirality Research Center, Chiba University
\(^4\) Graduate School of Science and Technology, Nara Institute of Science and Technology

**ABSTRACT**

Stress responses and pathogenicity have been extensively studied in *Aspergillus fumigatus*, the main causative pathogen of life-threatening aspergillosis. The heterogeneity in this pathogen’s biology has recently attracted increasing attention. In the present work, we used 16 clinically isolated strains to investigate several properties relevant to the pathogenicity of *A. fumigatus*, namely, gliotoxin production, elastase activity, hypoxia growth, adaptation to iron-limiting conditions, and growth upon nitrosative, oxidative, and high osmotic stresses. The range of phenotypes was diverse across the strains, with gliotoxin production and elastase activity being negatively correlated at an intermediate index (\(R = -0.4717\)). Notably, there were strains that showed extraordinary high production of gliotoxin or elastase activity and hypersensitivity to nitrosative or oxidative stresses. Clustering analysis showed that the 7 potentially pathogenicity-related phenotypes were not correlated with the genetic sub-group or pathotype. These results contribute to the growing awareness of the genetic and phenotypic diversity in *A. fumigatus* isolates.

**Key words**: *Aspergillus fumigatus*, clinical isolates, elastase activity, gliotoxin production, phenotypic relatedness

**Introduction**

*Aspergillus fumigatus* is the main causative fungal species for the group of diseases collectively termed aspergillosis. From a clinical perspective, aspergillosis is categorized into the following four types: invasive aspergillosis, aspergilloma, chronic necrotizing pulmonary aspergillosis (CNPA), and allergic bronchopulmonary aspergillosis\(^5\). In the lungs of patients with aspergilloma, the fungus exists as a fungus “ball” composed of hyphae, extracellular matrix, and host tissue debris.\(^6\) The fungal balls reside in the lung over several years, and can affect patient health when they are large enough. CNPA is regarded as a semi-invasive or slowly progressive form of aspergillosis. In both aspergilloma and CNPA, patients are typically moderately immunocompromised or suffer from chronic lung disease. Previously, we collected 17 *A. fumigatus* isolates from patients with aspergilloma or CNPA and compared their genome sequences\(^7\). Phylogenetic analysis using a profile of single nucleotide variants (SNVs) revealed three subgroups, but no correlation was observed between genotype and pathological condition in this small sample set.

Several studies have suggested that secretion of cytotoxic compounds impacts *A. fumigatus* pathogenicity and virulence\(^8\). Of the several factors that are thought to be secreted *in vivo*, gliotoxin shows relatively high toxicity to epithelial cells.\(^9\) Expression of the genes responsible for gliotoxin production is induced at an early stage (12-14 h after inoculation) in a mouse model of invasive aspergillosis\(^10\); and in a clinical setting, gliotoxin has been detected in patient samples including bronchoalveolar lavage fluid and sera.\(^7\) In steroid treated mice, loss of gliotoxin production has a modest impact on fungal virulence.\(^11\) In the lung, elastin is a major structural component of the tissue. Hence, elastase activity is considered to be relevant to the pathogenicity of *A. fumigatus*\(^12\).

In addition to secretion of compounds *in vivo*, the ability of the fungus to adapt to the host environment is a potential determinant of virulence and pathogenicity\(^13\). Recently, it was
reported that fitness in low oxygen was correlated with \textit{A. fumigatus} virulence and that fitness was heterogeneous across strains\textsuperscript{11}. Indeed, a strain that had evolved to adapt to low oxygen through \textit{in vitro} serial passaging in low-oxygen conditions showed increased virulence compared with the parental strain; moreover, the site of \textit{A. fumigatus} infection in mice reached oxygen tensions below 1%\textsuperscript{12}. Thus, the fungus must overcome this stress condition to successfully sustain disease progression\textsuperscript{15,16}. Iron limitation is another stress condition that the fungus encounters within the host\textsuperscript{15}. Siderophore production is induced in response to iron limitation, enabling \textit{A. fumigatus} to retrieve iron from its surrounding environment. The ability to produce siderophores is essential for \textit{A. fumigatus in vivo} growth and pathogenicity\textsuperscript{17,18}. During the progression of infection, the hyphae encounter immune cells such as resident macrophages and neutrophils. These host cells damage the invading hyphae with reactive oxygen and nitric oxide species\textsuperscript{19}. Tolerance to these stresses is a potential determinant of \textit{A. fumigatus} virulence and pathogenicity. However, our understanding of the scope of diversity in such virulence related attributes, and how this diversity may impact individual fungal-host interactions, remains limited for \textit{A. fumigatus}.

In the present study, we evaluated pathogenicity-related phenotypes, namely, gliotoxin production, elastase activity, low-oxygen growth, adaptation to iron limitation, and growth under nitrosative, oxidative, and osmotic stress conditions in a set of clinical isolates from patients with aspergilloma or CNPA. Using \textit{in vitro} phenotypic assays, we sought to determine whether the magnitude of such pathogenicity-related phenotypes is associated with the genetic relatedness and pathological condition of the strains.

Materials and methods

Fungal strains and media

The laboratory strain of \textit{A. fumigatus} Af293 was used as a reference. The 16 clinical isolates from patients with aspergilloma or CNPA were stored at Medical Mycology Research Center, Chiba University, Japan (serially numbered by IFM). The pathological conditions (aspergilloma and CNPA) were classified based on a definition suggested by Denning\textsuperscript{1}. The detailed strain information is described in our previous study\textsuperscript{3}. Because strains with different phenotypes appear to coexist in the stock of IFM 59361 (in fact, we had segregated IFM 59361-1 and IFM 59361-2 from the stock in a study by Hagiwara et al.\textsuperscript{11}), we used IFM 60237 instead of IFM 59361, both of which had been serially recovered from the same patient\textsuperscript{11}. RPMI-1640 containing 0.2 % glucose, L-glutamine, and phenol red (Wako Pure Chemical Industries, Osaka, Japan), Potato Dextrose Agar/Broth (PDA/PDB), glucose minimal medium (GMM), and Czapek Dox Agar (CDA) were used to culture the strains at 37°C. GMM is a conventional synthetic minimal medium for culturing \textit{Aspergillus} fungi, and is composed of 6 g NaNO\textsubscript{3}, 0.52 g KCl, 0.52 g MgSO\textsubscript{4}·7H\textsubscript{2}O, 1.52 g KH\textsubscript{2}PO\textsubscript{4}, 1 ml trace elements [2.2 g ZnSO\textsubscript{4}·7H\textsubscript{2}O, 1.1 g H\textsubscript{3}BO\textsubscript{3}, 0.5 g MnCl\textsubscript{2}·4H\textsubscript{2}O, 0.5 g FeSO\textsubscript{4}·7H\textsubscript{2}O, 0.16 g CoCl\textsubscript{2}·5H\textsubscript{2}O, 0.16 g CuSO\textsubscript{4}·5H\textsubscript{2}O, (NH\textsubscript{4})\textsubscript{6}Mo\textsubscript{7}O\textsubscript{24}·4H\textsubscript{2}O, 4H\textsubscript{2}O, 5 g Na\textsubscript{2}EDTA in 100 ml distilled H\textsubscript{2}O], 10 g glucose, 12.5 g agar, pH 6.5, in 1 liter distilled H\textsubscript{2}O\textsuperscript{11}.

Gliotoxin quantification

The conidia (final concentration, 10\textsuperscript{7} conidia/mL) of strains were inoculated in RPMI supplemented with 10% FBS and incubated at 37°C in a 5% CO\textsubscript{2} growth chamber for 2 d or 4 d. Two mL of the culture filtrates (CFs) were mixed with an equivalent volume of ethyl acetate and vigorously vortexed for 30 sec. The solutions were incubated for 10 min at room temperature, and the organic phase was collected in a new tube. The organic extracts were immediately dried by vacuum, and the precipitates were dissolved in 40 μL of methanol. Samples (10 μL) were injected into a high-performance liquid chromatography (HPLC) system using WakoSil-II, a 3C18HG column (particle size: 3 μ M; length: 150 mm; internal diameter: 3 mm) (Wako Pure Chemical Industries). The column was run using a gradient of 10%-100% acetonitrile in water over 45 min at a flow rate of 1 mL min\textsuperscript{-1} and monitored using a photodiode array detector (SPD-M10A VP, Shimadzu Corp., Kyoto, Japan). Gliotoxin production was quantified by obtaining peak areas detected by the absorbance at 254 nm in 3 biological replicates. A gliotoxin standard was purchased from Sigma-Aldrich (St. Luis, MO) and used to create a standard curve.

Elastin-Congo Red (ECR) agar plate assay

Production of elastase from \textit{A. fumigatus} was determined as described previously with a slight modification\textsuperscript{19}. Briefly, a stock solution of 20 mg/mL ECR (Nacalai Tesque, Kyoto, Japan) was prepared in 0.1 mM Tris-HCl (pH 7.4), and then added to 0.1% yeast carbon base (Difco Laboratories, Detroit, MI) and 1.5% agar (Wako Pure Chemical Industries) to give a final concentration of 2 μg/mL. Conidia (10\textsuperscript{7} in 1 μL) were inoculated onto the ECR agar plate, which was then incubated at 37°C for 3 d. Elastase activity was observed as a zone of clearing around the colony. The diameter of the colony and the halo of ECR lysis were measured, and the elastase activity index (EAI) was calculated by dividing the halo diameter by the colony diameter.

Hyoxic growth

Culture under hypoxic conditions was performed as described previously\textsuperscript{17}. Conidia of each strain were inoculated on GMM, and the plate was incubated in a multi-gas controlled incubator at 37°C, 5% CO\textsubscript{2}, and 0.2% O\textsubscript{2}. After 4 d, the colony diameters were measured, and the radial growth ratio (hypoxia/normoxia) was calculated for 3 biological replicates.
Iron-limitation growth test

Growth inhibition by an iron chelator was examined by performing a paper-disc diffusion assay. Conidia of each strain were mixed with 5 mL of CDA (final concentration, 10⁷ conidia/mL), poured into a 6-cm petri dish, and allowed to solidify. A paper disc was placed on the center of the plate, and 5 μL of 100 mM bipyridyl was dropped onto the disc. The plates were incubated at 37°C for 48 h before the inhibition halo diameter was measured. The mean diameter was calculated from 3 biological replicates.

Growth test for nitric oxide

Growth inhibition by nitric oxide (NO) was examined by using an NO donor, sodium nitroprusside (SNP). Conidia (10⁷) of each strain were inoculated on PDA and on PDA supplemented with 1 mM SNP. The plates were incubated at 37°C for 48 h before the colony diameter was measured. The growth rate in terms of + SNP/− SNP ratio was calculated for 3 biological replicates.

Growth inhibition by oxidative stress

Growth inhibition by hydrogen peroxide was examined by performing a paper-disc diffusion assay. Conidia of each strain were mixed with 5 mL of CDA (final concentration, 10⁷ conidia/mL), poured into a 6 cm petri dish, and allowed to solidify. A paper-disc was placed on the center of the plate, and 10 μL of 10% hydrogen peroxide solution (Nacalai Tesque) was dropped onto the disc. The plates were incubated at 37°C for 48 h before the inhibition halo diameter was measured. The mean diameter was calculated from 3 biological replicates.

Growth test with osmotic stress

Growth under osmotic stress conditions was examined by inoculating 10⁷ conidia of each strain on CDA and on CDA containing 1.2 M sorbitol. The plates were incubated at 37°C for 48 h before the colony diameter was measured. The growth rate in terms of + sorbitol/-sorbitol ratio was calculated for 3 biological replicates.

Clustering analysis

Phenotypic clustering was performed by using the Cluster 3.0 program with a hierarchical clustering setting, using a centered correlation for similarity metric and a centroid linkage method. The input dataset was prepared from quantitative values in each experiment, namely gliotoxin production [μg/ml CF], EAI, H/N ratio, diameters [mm] of inhibition halo for iron-limitation and hydrogen peroxide, growth rate upon SNP treatment, and growth rate upon sorbitol addition. The dendrogram was drawn using Java TreeView software.

Correlation analysis

Correlations were calculated using the Pearson’s correlation coefficient analysis for parametric data.

Results

Gliotoxin production and elastase activity of the A. fumigatus strains

The secretion of gliotoxin into CF was quantified in 16 A. fumigatus clinical isolates (8 strains from a patient with aspergilloma; 8 strains from another with CNPA) and the reference strain Af293 (originally isolated from a patient with invasive aspergillosis). The IFM 58401 strain showed the largest amount of gliotoxin at 14.42 μg/ml CF, whereas no gliotoxin was detected in the CF of 5 strains (Fig. 1A). We previously determined SNVs in the strains in reference to Af293. Here we dissected the SNVs within the gliotoxin biosynthesis gene cluster. SNVs were identified in a total of 16 positions among the strains: 3 reside in the 5’-UTR, 9 are in an ORF (7: nonsynonymous; 2: synonymous), and 5 are in an intergenic region (Supplemental Table S1). As depicted in Fig. 2, amino acid substitution in GliJ (V251I) and mutation in the 5’-UTR of gliF are specific to IFM 58026 strain, which produced no gliotoxin. Of note, the mutation in the 5’-UTR of gliF potentially generates a new start codon, which may lead to premature translation of gliF gene in the strain.

Elastase production was determined by the presence of zones of clearance on ECR agar (Fig. 1B). Plotting the values of gliotoxin production and EAI for all 17 strains revealed a moderate negative correlation between the two factors (R =−0.4717) (Fig. 1C). Notably, this negative correlation was much stronger in the subset of strains from the patient with aspergilloma (R =−0.9597).

Growth under low-oxygen conditions

We then assayed growth under oxygen-limited conditions. The oxygen concentration in the growth chamber was maintained at 0.2% for low-oxygen cultures, and the radial growth rate [hypoxia/normoxia (H/N ratio)] was examined. The 17 strains showed varied H/N ratios, of which IFM 55569, IFM 58401, IFM 60514, and IFM 61578 showed ratios less than 0.8 (Fig. 3A).

Growth inhibition by iron limitation, nitrosative, oxidative, and osmotic stresses

Adaptation to iron limitation was evaluated using an iron-chelating agent, bipyridyl. In a disc-diffusion assay, the smallest sized halo was observed with IFM 59777, indicating that the strain has the highest tolerance to iron limitation (Fig. 3B). Tolerance to nitrosative stress was evaluated using the nitric oxide donor SNP. The growth inhibition assay by an NO donor showed that adaptation to nitrosative stress varied across the isolates (Fig. 3C). Notably, IFM 60237 was unable to grow in the presence of 1 mM SNP, whereas the remaining strains grew moderately with rates ranging from 36% to 79% of the untreated controls. IFM 60237 was clearly hypersensitive to nitrosative stress compared with the isolates. Tolerance
to oxidative stress was quantified using a hydrogen peroxide disc-diffusion assay (Fig. 3D). The majority of the strains, including the reference strain Af293, showed around a 10 mm zone of inhibition, whereas IFM 61578 showed a 24.3 mm zone, revealing significant hypersensitivity in the strain. Under a high osmotic stress condition with 1.2 M sorbitol, IFM 60514 and IFM 61578 appeared tolerant, whereas IFM 61610 and IFM 62115 showed delayed growth compared with the rest (Fig. 3E).

**Genetic classification of the strains by cluster analysis of the phenotypes**

To gain insight into the phenotypic relatedness among the 17 strains used in this study, we performed a cluster analysis based on the pathogenicity-related phenotypes studied above (Fig. 4). The resulting phylogenetic tree consisted of three small clades (clade A: IFM 58026 (aspergilloma) and 62115 (CNPA); clade B: IFM 61118 (CNPA), 59365 (aspergilloma), and 62516 (CNPA); and clade C: IFM 59056 (aspergilloma), 61407 (CNPA), and 59073 (CNPA)) although the pathological condition mostly differed. Notably, IFM 58401 and IFM 61578 were distantly placed from the other strains.

In our previous study, the strains used here, with the exception of IFM 60237, had been grouped into 3 sub-groups (groups I, II, and III) based on the profile of the SNVs\(^3\). Groups I, II, and III included 4, 10, and 1 strains, respectively. Strains of each group were randomly distributed over the tree clustered based on the phenotypes (Fig. 3), suggesting that the genome-wide SNV profile was not consistent with the phenotypic classification.

**Discussion**

The Aspergilli are ubiquitous microorganisms that are able to cope with a variety of stressful conditions in their diverse niches. Several studies have investigated the responses and adaptations to such stresses at a molecular level, which have begun to illustrate the complex mechanisms used by these fungi to survive in diverse environments\(^{21}\). In the last decade, such research has focused on human pathogenic fungi, such as Candida and Cryptococcus, as well as A. fumigatus\(^{10, 17, 22}\). These studies have broadened the understanding of how these organisms cause disease. However, clinical isolates belonging to the same species are increasingly found to display significant genotypic and phenotypic heterogeneity\(^11, 23-26\). These data beg the questions: how do virulence and pathogenicity differ at the level of strains rather than species, and how does such information impact clinical approaches and outcomes, including diagnosis and treatment strategies? Indeed, Kowalski et al. demonstrated that the virulence of A. fumigatus was highly variable from strain-to-strain, and the
Table S1. SNVs found in gliotoxin biosynthesis gene cluster among the isolates

| Region       | Gene               | SNV type | Position (Chr. 6) | Alteration at the gene | Amino acid substitution | Isolates with SNV*1 |
|--------------|--------------------|----------|-------------------|------------------------|-------------------------|---------------------|
| 5'-UTR       | Afu6g09630         | Mutation | 2346922           | - A > C                | -                       | IFM 59359, IFM 58401 |
| ORF          | Afu6g09640         | Non-synonymous | 2350428       | 175 A > G               | T59A                   | All excepting IFM 62115 |
| 5'-UTR       | Afu6g09650         | Mutation | 2350772           | - 173 G > A            | -                       | IFM 59056            |
| ORF          | Afu6g09650         | Non-synonymous | 2351849       | 751 G > A               | V251I                  | IFM 58026            |
| ORF          | Afu6g09650         | Synonymous | 2352248        | 1150 T > C             | L384L                  | IFM 59056            |
| Intergenic   | Afu6g09650-09660   | Mutation | 2352338           | - T > C                | -                       | IFM 59056            |
| Intergenic   | Afu6g09650-09660   | Mutation | 2352513           | - G > A                | -                       | IFM 59056            |
| Intergenic   | Afu6g09650-09660   | Mutation | 2352528           | - G > T                | -                       | IFM 62115, IFM 59073, IFM 59777 |
| ORF          | Afu6g09660         | Non-synonymous | 2352723       | 6505 A > G             | S2102N                 | IFM 59359, IFM 55369, IFM 58401 |
| ORF          | Afu6g09660         | Non-synonymous | 2353201       | 5827 C > T             | P1943S                 | IFM 61578, IFM 61407, IFM 61118, IFM 59365, IFM 60514 |
| ORF          | Afu6g09660         | Non-synonymous | 2353504       | 5524 C > T             | R1842C                 | IFM 59365            |
| ORF          | Afu6g09660         | Non-synonymous | 2353555       | 5473 G > A             | A1825T                 | All 15 isolates       |
| ORF          | Afu6g09660         | Non-synonymous | 2357213        | 1865 A > C             | H622P                  | All excepting IFM 59056 |
| Intergenic   | Afu6g09660-09670   | Mutation | 2359549           | - A > G                | -                       | IFM 59056, IFM 59359, IFM 61578, IFM 58401, IFM 61407, IFM 61118, IFM 59365, IFM 58026, IFM 61610, IFM 60514 |
| Intergenic   | Afu6g09660-09670   | Mutation | 2359624           | C > T                  | -                       | All excepting IFM 62115 |
| 5'-UTR       | Afu6g09730         | Mutation | 2371144           | - 207 C > T            | -                       | IFM 58026            |

*1: The IDs whose isolate did not produce gliotoxin in this study are indicated by bold style.

Fig. 2. SNVs in the gliotoxin biosynthesis gene cluster.

The gli genes are indicated by black arrows showing their direction on the genome. The mutations in the 5'-UTR are indicated by black triangles, and nonsynonymous mutations are indicated by white triangles. Nucleotides and amino acids are presented in lower and upper cases, respectively. The SNVs that are different from Af293 are depicted in white letters. The strains that did not produce gliotoxin are represented by an asterisk before the strain number and are underlined.
The virulence of these individual strains was correlated with the magnitude of their ability to grow under an in vitro low-oxygen condition. In addition to low-oxygen growth, a separate subset of clinical isolates revealed heterogeneity in conidia pigmentation and the accumulation of the cytotoxic compound, trypacidin. Very recently, Takahashi-Nakaguchi et al. observed diversity in the adherence of *A. fumigatus* conidia to the A549 epithelial cell line, which may impact virulence across the strains. In the context of these studies, we here evaluated the phenotypic diversity of 16 *A. fumigatus* clinical isolates in terms of their pathogenicity-related attributes, including gliotoxin production, elastase activity, low-oxygen growth, iron-limited growth, and tolerance to nitrosative, oxidative, and high osmotic stresses.

The 16 clinical isolates, as well as Af293, displayed a range of phenotypes—a result that was not unexpected given the aforementioned studies on the natural heterogeneity in this species. We found, for example, that IFM 60514 displayed more than twice the elastase activity of Af293, and IFM 58401 produced a large amount of gliotoxin that was more than 4 times that detected in Af293. In addition, IFM 60237 showed no growth in the presence of nitrosative stress, and IFM 61578 was hypersensitive to oxidative stress. Identifying such strains with irregular phenotypes offers a unique opportunity to investigate the molecular mechanisms underlying stress resistance.
tolerance and pathogenesis through whole genome sequence analysis. Indeed, SNVs were identified in the gliotoxin biosynthesis gene cluster of IFM 58026, which might cause inability of the strain to produce gliotoxin. Recent advances in population-genomics will allow the characterization of novel aspects of stress biology, which remains a focus of our future work.

One notable finding in this study is a moderate negative correlation between gliotoxin production and EAI. Elastin is one of the major components of human elastic tissues such as lung, and several studies have revealed a connection between elastin degradation and *A. fumigatus* invasiveness. In fact, Garcia et al. reported an increase in the number of strains with EAI > 1 isolated from successively inoculated mice using an initial environmental strain with no EAI. Similarly, a correlation of gliotoxin production and pathogenicity in insects was evaluated by Reeves et al.; where an *A. fumigatus* strain with increased gliotoxin production was found to be more virulent. Although the mechanism underlying this correlation between elastase activity and gliotoxin production remains elusive, there might be a strain-dependent preference of metabolism or secretion in elastase and gliotoxin production. Given the small number of strains analyzed, further research is needed to understand the potential mechanistic relationship between gliotoxin and elastase activity and whether this relationship exists in vivo and impacts disease outcomes.

In conclusion, our data support the growing evidence that significant genotypic and phenotypic diversity exists across *A. fumigatus* isolates. While we did not observe a correlation with these attributes and in vivo disease phenotypes, larger sample sizes are needed to better understand the relationship between strain diversity and clinical outcomes. However, the growing number of genotype-phenotype datasets for this fungus will eventually lead to the elucidation of more causal relationships that will enhance our understanding of *A. fumigatus* pathogenesis mechanisms.

**Author contributions**

Conceived and designed the experiments: DH
Performed the experiments: DH
Analyzed the data: DH, HTakahashi, HTakagi, AW, KK
Contributed reagents/materials/analysis tools: HTakahashi, AW, KK
Wrote the paper: DH

**Acknowledgement**

We appreciate Caitlin H. Kowalski and Robert A. Cramer, Geisel School of Medicine at Dartmouth for their contribution to the data collection.

**Funding**

This work was supported by AMED under Grant Number JP18fm0208024 (D. H., H. Takahashi, and A. W.) and 18im0110015 (D.H., H. Takahashi, A.W., and K.K.), and in part by a Grant-in-Aid for Scientific Research (A) (25252065) from the Japan Society for the Promotion of Science (D.H. and H. Takagi) and by a grant from the Institute for Fermentation, Osaka (D.H. and H. Takagi)

**Disclosure of conflict of interest**

No conflict of interest.

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