RESEARCH ARTICLE

Temperature during conidiation affects stress tolerance, pigmentation, and trypacidin accumulation in the conidia of the airborne pathogen *Aspergillus fumigatus*

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Abstract

Asexual spores (conidia) are reproductive structures that play a crucial role in fungal distribution and survival. As fungal conidia are, in most cases, etiological agents of plant diseases and fungal lung disease, their stress resistance and interaction with their hosts have drawn increasing attention. In the present study, we investigated whether environmental temperature during conidiation affects the stress tolerance of the conidia of the human pathogenic fungus *Aspergillus fumigatus*. Conidia from a 25˚C culture showed a lower tolerance to heat (60˚C) and oxidative (H₂O₂) stresses and a marked resistance to ultraviolet radiation exposure, compared with those produced at 37 and 45˚C. The accumulation of trehalose was lower in the conidia from the 25˚C culture. Furthermore, the conidia from the 25˚C culture showed darker pigmentation and increased transcripts of dihydroxynaphthalene (DHN)-melanin biosynthesis-related genes (i.e., *pksP*, *arp1*, and *arp2*). An RNA-sequencing analysis revealed that the transcription level of the trypacidin (*tpc*) gene cluster, which contains 13 genes, was sharply and coordinately activated in the conidia from the 25˚C culture. Accordingly, trypacidin was abundant in the conidia from the 25˚C culture, whereas there was little trypacidin in the conidia from the 37˚C culture. Taken together, these data show that the environmental temperature during conidiation affects conidial properties such as stress tolerance, pigmentation, and mycotoxin accumulation. To enhance our knowledge, we further explored the temperature-dependent production of DHN-melanin and trypacidin in clinical *A. fumigatus* isolates. Some of the isolates showed temperature-independent production of DHN-melanin and/or trypacidin, indicating that the conidia-associated secondary metabolisms differed among the isolates.
Introduction

Filamentous fungi vigorously produce asexual spores (conidia) under appropriate conditions. Conidia are reproductive structures that play a crucial role in the distribution and survival of fungi [1]. In general, *Aspergillus* conidia are relatively stress-resistant cells that survive environmental stresses such as drought, high temperatures, and ultraviolet (UV) irradiation [2,3]. Fungi protect themselves from such abiotic stresses by accumulating compatible solutes. Mannitol and trehalose are the most prevalent solute sugars that accumulate in conidia. While mannitol is the most abundant solute in *Aspergillus oryzae* and *Aspergillus niger* conidia, trehalose is the major compatible solute that accumulates in *Aspergillus fumigatus* conidia [4–6]. The absence of the compatible solutes reduces the heat resistance and/or longevity of *A. niger* and *Aspergillus nidulans* conidia [2,7].

*A. fumigatus* is the causative agent of aspergillosis, which encompasses several types of infection, including life-threatening invasive aspergillosis. Conidia are the main infectious agents, and they are inhaled daily because they are ubiquitous in the environment [8,9]. The conidia of *A. fumigatus* have dihydroxynaphthalene (DHN)-melanin in their cell wall, which is thought to provide structural rigidity and protection from environmental stresses, such as UV irradiation and heat [10]. A genetic study highlighted the crucial role of DHN-melanin in *A. fumigatus* virulence, because the virulence of a polyketide synthase (PKS) mutant, *pksP*, which fails to produce the PKS that is responsible for DHN-melanin synthesis, was attenuated in a murine infection model of disseminated aspergillosis [11]. Furthermore, DHN-melanin protects conidia against phagocytic killing by inhibiting the acidification of phagolysosomes [12,13]. Additionally, pigment-less, white conidia of a *pksP* mutant were ingested more frequently compared with wild-type (WT) conidia by the soil-living amoeba *Dictyostelium discoideum* [14]. Thus, DHN-melanin not only plays an essential role in infections of mammalian hosts but also contributes to avoiding predation by amoeba. Different types of melanin (Asp-melanin) on the conidia of *Aspergillus terreus* also inhibit phagocytosis by *D. discoideum* [15], which supports the importance of melanin for fungal conidia to survive in their environments.

*A. fumigatus* is also a major airborne allergen, and it possesses 22 known allergenic proteins. Temperature impacts the allergenicity and viability of *A. fumigatus* conidia [16]. An increased level of the Asp f1 major allergen was found in conidia collected from cultures grown at 17˚C, compared with those grown at 32˚C. Environmental conditions during conidiation affect the physiological properties, such as morphology, germination kinetics, and pathogenicity, of spores from other fungi [17–20]. In addition, entomopathogenic fungi have demonstrated that virulence toward insect hosts, and conidial tolerance to heat and UV radiation, are greatly influenced by environmental conditions such as the medium, water activity, light illumination, and oxygen concentration, during conidia production [21,22]. Thus, environmental conditions appear to be key factors that affect fungal conidial properties; however, little is known about the impacts of temperature on the properties of the conidia of *Aspergillus* species, including the medically important pathogenic fungus *A. fumigatus*.

Here, we investigated the effects of temperature during conidia production on the stress resistance of *A. fumigatus* conidia. Based on a comparative transcriptome analysis, we found that conidia from a 25˚C culture accumulated greater amounts of DHN-melanin and the cytotoxic secondary metabolite trypacidin, compared with those from a 37˚C culture.

Results

Conidia harvested from cultures grown at different temperatures show divergent stress resistances

To examine the impact of temperature on conidial physiology, we cultivated the *A. fumigatus* Af293 strain on potato dextrose agar (PDA) for 7 d at 25, 37, and 45˚C, after which the conidia
were collected. We investigated how the conidia tolerated short bursts of heat stress (60°C for 15 min), oxidative stress [200 mM hydrogen peroxide (H₂O₂) for 15 min], and UV stress (9.55 Wm⁻² of UV light for 1 min) that are encountered by fungal conidia in nature. The conidia from the 25°C culture showed a significantly greater sensitivity to heat stress, compared with those from the 37 and 45°C cultures (Fig 1A). Upon H₂O₂ treatment (as an oxidative stressor), the conidia from the 25°C culture were more sensitive than those from the 37°C culture, whereas the conidia from the 45°C culture showed a greater stress tolerance (Fig 1B). In contrast, the conidia from the 25°C culture showed a marked tolerance to UV irradiation (Fig 1C). To rule out the possibility that the conidial maturation levels affected the differences in phenotypes, we compared the heat-stress tolerance of the conidia harvested from the 25 and 37°C cultures in a time-dependent manner (Fig 1D). The conidia from the 25°C culture were more sensitive to heat stress than those from the 37°C at all of the culture ages (3d, 7d, and 14d). Thus, the conidiation temperature affected the stress tolerance of *A. fumigatus* conidia.

**Reduced trehalose levels in conidia from a 25°C culture**

Trehalose is the major compatible solute in *A. fumigatus* conidia that protects against environmental stresses, including heat and oxidation [5,23]. Thus, we examined the amounts of carbohydrates that accumulated in conidia from cultures grown at 25, 37, and 45°C. The conidia from the 25°C culture exhibited a lower level of trehalose than those from the 37 and 45°C cultures (Fig 2A). This reduction was assumed to cause the heat and oxidative stress-labile phenotypes of the conidia from the 25°C culture. Notably, the conidia from the 25°C culture accumulated an abundant amount of arabitol, whereas the conidia grown at the other temperatures exhibited only low levels of arabitol. This may be a compensating metabolic effect, but its role in the aforementioned stress tolerance remains unknown.

To understand the mechanism underlying the lower trehalose level in the conidia from the 25°C culture, transcript levels of trehalose metabolism-related genes, including the trehalose-6-phosphate synthases *tpsA* to *tpsD*, the trehalose-6-phosphate phosphatase *orlA*, and the trehalases *treA*, and *treB* were investigated (Fig 2B). The conidia from the 25°C culture accumulated greater amounts of *treB* transcripts compared with those from the 37°C culture, suggesting the more efficient degradation of trehalose in the conidia. However, the higher transcript levels of *tpsD* in the conidia from 25°C culture were shown, which might promote trehalose production.

**DHN-melanin accumulates at high levels in conidia from a 25°C culture**

We compared the structural properties of conidia isolated from cultures that were grown at different temperatures. Under microscopic observation, the size and form of the conidia were comparable among all of the cultures (data not shown). However, the pigmentation level differed, as the conidia from the 25°C culture exhibited a darker, greenish-gray color (Fig 3A), which indicates the presence of DHN-melanin on their surface. To determine whether the pigmentation in the conidia from the 25°C culture was caused by DHN-melanin, we used tricyclazole (TCZ), a specific inhibitor of DHN-melanin biosynthesis [24]. In the presence of TCZ, the conidia were brown, and the 25°C culture was a darker brown than the 37°C culture (Fig 3B). This suggests that a lower temperature during conidiation promoted a higher level of DHN-melanin accumulation in the conidia. We also confirmed pigmentation levels of the conidia in a series of culture ages, with the conidia from 5, 7, and 10 d of growth at 25°C revealing dense pigmentation compared with those harvested from the culture at 37°C (Fig 3C). This indicated that culture age did not affect the pigmentation levels but the temperature did.

DHN-melanin is synthesized through a pathway that includes the proteins PksP, Ayg1, Arp2, Arp1, Abr1, and Abr2 during conidiogenesis and conidial maturation [10]. Thus, we
investigated the transcriptional levels of the corresponding genes between conidia that were grown at 25 and 37˚C. The transcripts of *pksP*, which encodes a PKS that is involved in the first step of the pathway, were four times more abundant in the conidia from the 25˚C culture than in those from the 37˚C culture (Fig 3D). *arp1* and *arp2* were also expressed at higher levels in the conidia from the 25˚C culture, which confirmed that the DHN-melanin biosynthesis pathway was highly expressed in conidia grown at 25˚C, and this was likely responsible for the abundance of DHN-melanin on their surfaces.

Comparing transcriptomes among conidia produced at different temperatures

To gain a broader insight into the physiological impact of temperature on conidia, we conducted an RNA sequencing analysis to generate genome-wide transcriptional profiles. The

![Figure 1. Comparison of conidial stress resistance.](https://doi.org/10.1371/journal.pone.0177050.g001)
Fig 2. Polyols and trehalose contents of conidia. (A) Polyols and trehalose were extracted from conidia and analyzed by high-performance liquid chromatography (HPLC). Error bars represent the standard deviation based on six independent replicates. (B) Expression analysis of trehalose metabolism related genes in the conidia from the 25 and 37°C cultures. RNA was extracted from Af293 conidia that were produced at the indicated temperature. The expression of tpsA-D, orlA, and treA,B was determined by quantitative real-time polymerase chain reaction (qRT-PCR). Each sample was tested in three biological replicates. Error bars represent the standard deviation. Statistical analysis (Student’s t-test) was performed, and significant differences were indicated as an asterisk (**: p<0.01; *: p<0.05).

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RNAs were extracted from resting conidia that were collected from cultures that were grown on PDA for 7 d at 25, 37, and 45˚C (see the Material and Methods). The whole transcriptome data is provided in S3 Table. Transcripts of 8,953 genes were detected in the conidia from the 25, 37, and 45˚C cultures. Among them, 492 and 696 genes showed greater than 4-fold increases in transcript levels in the conidia from the 25 and 45˚C cultures, respectively, compared with those from the 37˚C culture. Among these, the genes with enriched transcripts [showing more than 25% of mean fragments per kilobase of transcript per million mapped reads (FPKM) value and >10-fold increased] are listed in Table 1. The genes whose transcripts were up-regulated in the conidia from the 45˚C culture included those encoding a non-ribosomal peptide synthase (NRPS)-like enzyme (AFUA_8G01640) and a GPI-anchored cell wall organization protein Ecm33. On the contrary, the increased expression levels of rodA, ayg1, and conF, as well as trypacidin biosynthesis genes were found in the conidia from the 25˚C culture. The transcript levels of the allergen-related Asp f genes are listed in S2 Table. In total, 11, 10, and 10 out of the 22 Asp f genes showed higher FPKM values than the mean FPKMs (113.0, 103.3, and 93.7) in the conidia from the 25, 37, and 45˚C cultures, respectively. However, no obvious trend in the temperature-dependent transcriptional changes was found.

**Temperature-dependent synthesis of trypacidin in conidia**

According to Sanchez et al. [25] and the *Aspergillus* Genome Database (AspGD) annotations, *A. fumigatus* possesses 18 (putative or validated) NRPS and NRPS-like genes, 15 PKS and
### Table 1. FPKM data for the genes that were enriched at different conidiation temperatures.

| Gene ID | FPKM | Conidia from 25°C | Conidia from 37°C | Conidia from 45°C | 25°C/37°C | 45°C/37°C | Gene name | Annotation |
|---------|------|-------------------|-------------------|-------------------|-----------|-----------|-----------|-----------|
| **Enriched in 45°C conidia** | | | | | | | | |
| AFUA_7G00150 | 0.6 | 1.6 | 48.0 | 0.37 | 30.24 | nscC | FAD-dependent monoxygenase, putative |
| AFUA_1G01080 | 2.4 | 1.7 | 42.3 | 1.40 | 25.06 | conserved hypothetical protein |
| AFUA_5G09020 | 4.8 | 2.7 | 49.3 | 1.73 | 17.96 | wsc3 | ER membrane protein Wsc4, putative |
| AFUA_3G13570 | 10.7 | 2.7 | 37.7 | 4.00 | 14.08 | conserved hypothetical protein |
| AFUA_7G01470 | 5.4 | 4.4 | 61.0 | 1.24 | 14.02 | integral membrane protein, Mpv17/PMP22 family, putative |
| AFUA_7G01210 | 11.6 | 2.1 | 28.8 | 5.60 | 13.87 | conserved hypothetical protein |
| AFUA_1G05890 | 13.7 | 2.1 | 28.5 | 6.54 | 13.59 | hypothetical protein |
| AFUA_5G09020 | 4.8 | 2.7 | 49.3 | 1.73 | 17.96 | wsc3 | ER membrane protein Wsc4, putative |
| AFUA_1G06200 | 5.5 | 4.7 | 54.0 | 1.17 | 11.54 | Mn2+ homeostasis protein (Per1), putative |
| AFUA_3G02640 | 3.6 | 3.5 | 39.7 | 1.05 | 11.47 | nucleoside-diphosphate-sugar epimerase family protein |
| AFUA_4G00640 | 56.5 | 0.4 | 1.9 | 126.97 | 4.25 | macrophomate synthase, putative |
| AFUA_1G01980 | 3039.2 | 31.0 | 34.3 | 97.92 | 1.11 | IgE binding protein, putative |
| AFUA_5G07420 | 98.9 | 2.2 | 3.1 | 44.35 | 1.38 | hypothetical protein |
| AFUA_5G00660 | 502.9 | 11.6 | 1.4 | 43.21 | 0.12 | hypothetical protein |
| AFUA_4G14580 | 1132.4 | 28.0 | 12.3 | 40.48 | 0.44 | tpcA | O-methyltransferase, putative |
| AFUA_4G14490 | 799.4 | 20.4 | 4.3 | 39.10 | 0.21 | tpcJ | extracellular dihydrogeodi n oxidase/laccase, putative |
| AFUA_4G14570 | 1091.1 | 28.3 | 7.3 | 38.52 | 0.26 | tpcB | metallo-beta-lactamase domain protein, putative |
| AFUA_5G09030 | 35.9 | 1.0 | 0.8 | 36.65 | 0.85 | F-box domain and ankyrin repeat protein |
| AFUA_4G14520 | 509.1 | 14.3 | 11.7 | 35.67 | 0.82 | tpcG | toxin biosynthesis protein, putative |
| AFUA_4G14460 | 2001.8 | 58.5 | 21.4 | 34.24 | 0.37 | tpcM | conserved hypothetical protein |
| AFUA_4G14510 | 3137.1 | 95.0 | 23.8 | 33.01 | 0.25 | tpcH | hypothetical protein |
| AFUA_8G06890 | 101.5 | 3.2 | 7.3 | 32.02 | 2.29 | extracellular exo-polygalacturonase, putative |
| AFUA_4G14480 | 1253.4 | 40.1 | 7.5 | 31.26 | 0.19 | tpcL | conserved hypothetical protein |
| AFUA_4G14470 | 1073.0 | 34.5 | 8.5 | 31.08 | 0.25 | tpcK | hypothetical protein |
| AFUA_5G01260 | 572.4 | 19.4 | 6.0 | 29.54 | 0.31 | ankyrin repeat protein |
| AFUA_4G14560 | 270.6 | 9.3 | 4.5 | 29.01 | 0.48 | tpcC | polyketide synthase, putative |
| AFUA_4G14500 | 454.7 | 15.7 | 3.2 | 28.91 | 0.20 | tpcI | conserved hypothetical protein |
| AFUA_2G12690 | 118.1 | 4.6 | 3.9 | 25.68 | 0.85 | hypothetical protein |
| AFUA_2G00140 | 33.0 | 1.4 | 2.8 | 24.41 | 2.06 | FAD monoxygenase, putative |
| AFUA_7G06820 | 99.8 | 4.2 | 1.4 | 23.91 | 0.33 | galactose oxidase, putative |
| AFUA_2G00150 | 96.2 | 4.2 | 2.4 | 23.03 | 0.57 | catecholamine-O-methyltransferase, putative |
| AFUA_1G05910 | 2155.0 | 99.0 | 85.0 | 21.78 | 0.86 | conserved hypothetical protein |
| AFUA_4G14530 | 1173.8 | 56.9 | 64.5 | 20.62 | 1.13 | tpcF | glutathione S-transferase Ure2-like, putative |
| AFUA_4G14540 | 151.9 | 7.9 | 15.3 | 19.33 | 1.95 | tpcE | C6 transcription factor (AllR), putative |
| AFUA_4G14550 | 434.8 | 22.5 | 51.6 | 19.29 | 2.29 | tpcD | toxin biosynthesis regulatory protein AfuJ, putative |
| AFUA_8G01210 | 75.6 | 4.4 | 3.2 | 17.32 | 0.28 | enoyl-CoA hydratase/isomerase family protein |
| AFUA_3G14260 | 2943.7 | 174.2 | 125.2 | 16.90 | 0.72 | mismatched base pair and cruciform DNA recognition protein, putative |
| AFUA_7G05100 | 64.8 | 3.9 | 15.5 | 16.69 | 3.98 | hexose transporter protein |
| AFUA_3G13130 | 87.4 | 5.3 | 11.4 | 16.60 | 2.17 | HHE domain protein |

(Continued)
PKS-like genes, and one PKS-NRPS hybrid gene. The expression profiles of the NRPS and PKS genes are listed in Table 2. Among these, one NRPS-like gene (AFUA_8G01640) and two PKS genes [\textit{pksP} and \textit{tpcC} (also called \textit{tynC})] were found to be relatively highly expressed in the conidia. AFUA_8G01640 was more highly expressed in the conidia that were grown at 45°C, whereas \textit{pksP} and \textit{tpcC/tynC}, which are responsible for DHN-melanin and trypacidin biosynthesis, respectively, were more highly expressed at 25°C. The trypacidin biosynthesis (\textit{tpc/tyn}) gene cluster was recently reported to comprise 13 contiguous genes (\textit{tpcA/tynA} to \textit{tpcM/tynM}) [26,27]. Indeed, the present RNA sequencing analysis showed that the expression of the \textit{tpc} genes was coordinately regulated in a temperature-dependent manner, with clear borders on both ends of the gene cluster (Fig 4A).

We also performed a motif-independent, \textit{de novo} detection of secondary metabolite gene clusters using the MIDDAS-M program [28]. In addition to the \textit{tpc} cluster, this analysis revealed that the expression levels of two additional sets of genes were coordinately regulated in a conidiation temperature-dependent manner (Fig 4B). Both sets of genes were expressed at lower levels in the conidia from the 45°C culture, compared with those from the 37°C culture (S2 Table). One cluster contained four genes, including a gene encoding a predicted 1,4-alpha-glucosidase, whereas the other contained five genes, including \textit{pr1}, which encodes a putative

| Gene ID       | FPKM   | Conidia from 25°C | Conidia from 37°C | Conidia from 45°C | 25°C/37°C | 45°C/37°C | Gene name                     | Annotation                              |
|---------------|--------|------------------|------------------|------------------|----------|----------|-------------------------------|-----------------------------------------|
| AFUA_4G03010  | 930.8  | 56.6            | 57.9             | 16.44            | 1.02     |          | conserved hypothetical protein |
| AFUA_7G06840  | 1858.2 | 114.1           | 53.6             | 16.29            | 0.47     |          | class III aminotransferase, putative |
| AFUA_1G11560  | 71.4   | 4.7             | 15.9             | 15.23            | 3.40     |          | 4-hydroxyphenylpyruvate dioxygenase, putative |
| AFUA_6G14510  | 138.1  | 9.6             | 16.5             | 14.44            | 1.72     |          | monoxygenase, putative         |
| AFUA_6G01980  | 41.2   | 2.9             | 14.9             | 14.40            | 5.22     |          | haemolysin-III family protein  |
| AFUA_6G09570  | 403.6  | 28.3            | 23.2             | 14.24            | 0.82     |          | conserved hypothetical protein |
| AFUA_5G09580  | 1436.6 | 103.9           | 108.3            | 13.82            | 1.04     |          | RodA                          |
| AFUA_7G06830  | 240.8  | 17.4            | 11.6             | 13.82            | 0.66     |          | MFS transporter, putative      |
| AFUA_8G07260  | 42.8   | 3.1             | 3.1              | 13.69            | 0.99     |          | conserved hypothetical protein |
| AFUA_8G04920  | 201.1  | 14.9            | 8.2              | 13.49            | 0.55     |          | LEA domain protein             |
| AFUA_7G00950  | 285.3  | 22.1            | 26.7             | 12.89            | 1.21     |          | MFS monosaccharide transporter, putative |
| AFUA_5G00145  | 101.3  | 8.1             | 2.5              | 12.48            | 0.31     |          | conserved hypothetical protein |
| AFUA_4G00730  | 3748.9 | 308.7           | 61.8             | 12.14            | 0.20     |          | HHE domain protein             |
| AFUA_3G14750  | 31.9   | 2.6             | 1.3              | 12.08            | 0.48     |          | fungal specific transcription factor, putative |
| AFUA_2G11870  | 116.2  | 10.0            | 21.4             | 11.59            | 2.13     | kre6    | beta-1,6 glucan synthetase (Kre6), putative |
| AFUA_7G08320  | 48.8   | 4.2             | 10.8             | 11.51            | 2.55     |          | heat shock transcription factor, putative |
| AFUA_7G01720  | 647.0  | 56.9            | 95.9             | 11.36            | 1.68     |          | 3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase |
| AFUA_2G17550  | 208.0  | 18.5            | 6.2              | 11.24            | 0.34     | ayg1    | conidial pigment biosynthesis protein Ayg1 |
| AFUA_1G16960  | 2413.7 | 215.9           | 310.3            | 11.18            | 1.44     |        | conserved hypothetical protein |
| AFUA_4G08170  | 979.3  | 92.1            | 68.5             | 10.64            | 0.74     | uga2    | succinate-semialdehyde dehydrogenase Uga2, putative |
| AFUA_1G13800  | 507.9  | 48.3            | 23.4             | 10.52            | 0.48     |        | MFS multidrug transporter, putative |
| AFUA_1G05920  | 69.5   | 6.8             | 10.8             | 10.20            | 1.59     |        | conserved hypothetical protein |
| AFUA_6G08780  | 128.8  | 12.7            | 22.3             | 10.11            | 1.75     |        | proline utilization protein PrnX, putative |
| AFUA_4G03615  | 7668.7 | 764.5           | 266.3            | 10.03            | 0.35     | conF    | conidiation protein Con-6, putative |

Table 1. (Continued)

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alkaline serine protease. The two sets of genes did not appear to be involved in secondary metabolism. This MIDDAS-M analysis confirmed the temperature-dependent transcriptional activation of the tpc cluster.

To investigate whether trypacidin production was affected by the conidiation temperature, we analyzed extracts of conidia from a WT strain and a tpcC deletion mutant (designated ΔtpcC) that were cultured at 25 and 37˚C (Fig 5). We found that two peaks in the Af293

### Table 2. FPKM data for secondary metabolism genes.

| Gene ID | Conidia from 25˚C | Conidia from 37˚C | Conidia from 45˚C | Ratio 25˚C/37˚C | Ratio 45˚C/37˚C | Gene Predicted or actual products |
|---------|-------------------|-------------------|-------------------|----------------|----------------|----------------------------------|
| NRPS or NRPS-like |
| AFUA_1G10380 | 5.1 | 0.8 | 0.9 | 6.01 | 1.06 | pes1/nrps1 Fumigaclavine C |
| AFUA_1G17200 | 3.4 | 1.8 | 4.0 | 1.91 | 2.23 | sidC Ferricrocin, hydroxyferricrocin |
| AFUA_3G02670 | 17.9 | 6.5 | 27.0 | 2.77 | 4.17 | |
| AFUA_3G03350 | 0.3 | 0.2 | 0.9 | 1.40 | 3.61 | sidE |
| AFUA_3G03420 | 9.7 | 0.4 | 0.1 | 25.99 | 0.14 | sidD Fusaricine C, triacetylfusaricine C |
| AFUA_3G12920 | 0.1 | 0.9 | 1.7 | 0.12 | 1.93 | hasD Hexadehydroastechrome |
| AFUA_3G13730 | 3.7 | 2.6 | 1.1 | 1.40 | 0.42 | pesG |
| AFUA_3G15270 | 0.1 | 0.0 | 0.1 | - | - | pesH |
| AFUA_5G10120 | 5.9 | 6.1 | 18.8 | 0.96 | 3.07 | |
| AFUA_5G12730 | 43.2 | 28.4 | 12.0 | 1.52 | 0.42 | pes3 |
| AFUA_6G03480 | 0.4 | 0.4 | 1.5 | 0.93 | 3.40 | |
| AFUA_6G08560 | 5.2 | 4.1 | 15.7 | 1.27 | 3.81 | |
| AFUA_6G09610 | 2.1 | 0.3 | 2.0 | 6.22 | 5.95 | pesJ |
| AFUA_6G09660 | 1.2 | 0.4 | 5.4 | 2.80 | 12.74 | gliP Gliotoxin |
| AFUA_6G12050 | 11.0 | 3.2 | 7.8 | 3.38 | 2.40 | fmgC Fumiquinazolines |
| AFUA_6G12080 | 2.0 | 0.7 | 0.9 | 2.76 | 1.31 | fmgA Fumiquinazolines |
| AFUA_8G00170 | 0.3 | 0.1 | 0.8 | 5.60 | 13.59 | fmaPKS FumitremorginS |
| AFUA_8G01640 | 0.5 | 180.0 | 2370.4 | 0.00 | 13.17 | |
| PKS or PKS-like |
| AFUA_1G01010 | 0.0 | 0.1 | 0.4 | 0.00 | 2.97 | |
| AFUA_1G17740 | 1.2 | 1.9 | 9.3 | 0.64 | 4.93 | |
| AFUA_2G01290 | 6.7 | 6.0 | 8.8 | 1.13 | 1.47 | |
| AFUA_2G17600 | 207.3 | 74.9 | 18.0 | 2.77 | 0.24 | pksP DHN-melanin |
| AFUA_3G01410 | 8.4 | 8.1 | 20.0 | 1.03 | 2.46 | |
| AFUA_3G02530 | 5.5 | 5.9 | 7.8 | 0.93 | 1.33 | |
| AFUA_3G02570 | 7.2 | 6.5 | 9.2 | 1.10 | 1.41 | |
| AFUA_3G14700 | 0.3 | 0.1 | 0.5 | 3.27 | 5.10 | |
| AFUA_4G00210 | 2.4 | 1.6 | 4.3 | 1.49 | 2.70 | encA Endochrocin |
| AFUA_4G14560 | 270.6 | 9.3 | 4.5 | 29.01 | 0.48 | tpcC |
| AFUA_6G13930 | 0.3 | 1.2 | 1.5 | 0.25 | 1.27 | |
| AFUA_7G00160 | 3.3 | 11.4 | 34.6 | 0.29 | 3.04 | nscA/fccA Neosartorinc/fumicycline A |
| AFUA_8G00370 | 0.5 | 0.3 | 0.6 | 1.71 | 1.98 | fma-PKS Fumagillin |
| AFUA_8G00490 | 0.0 | 0.1 | 4.7 | 0.00 | 36.52 | |
| AFUA_8G02350 | 2.0 | 3.0 | 6.3 | 0.69 | 2.12 | |
| PKS-NRPS hybrid |
| AFUA_8G00540 | 0.5 | 0.2 | 1.0 | 2.99 | 6.46 | psoA Pseurotin |

https://doi.org/10.1371/journal.pone.0177050.t002
conidia from the 25˚C culture were not seen in the mutant or in the Af293 conidia from the 37˚C culture. The compounds corresponding to peaks with retention times (RTs) of 18.5 and 19.7 min were isolated from the Af293 conidia from the 25˚C culture. Their structures were identified by mass spectroscopy and a NMR analysis as trypacidin (RT: 18.5) and monomethylsulochrin (RT: 19.7). Monomethylsulochrin is a biosynthetic intermediate of trypacidin, and the production level of trypacidin was significantly lower in Af293 conidia from the 37˚C culture compared with that in the conidia from the 25˚C culture (Fig 6A). Thus, the tpc cluster was exclusively and highly activated at 25˚C during conidiation.

Temperature-dependent trypacidin and DHN-melanin production differ in clinical strains

To broaden our knowledge of the temperature-dependent accumulation of trypacidin, we investigated the ability of eight clinical strains, which were isolated from patients with chronic...
pulmonary aspergillosis, to produce trypacidin. Trypacidin quantification showed that the strains IFM 59073 and Af293 contained significantly higher amounts of trypacidin in the conidia harvested from 25˚C cultures compared to those from 37˚C cultures (Fig 6A). More interestingly, the IFM 59365 conidia accumulated abundant trypacidin, even when they were cultured at 37˚C (Fig 6A). Then, the relative level of the $tpcC$ transcript was determined in the conidia of the clinical isolates. All of the isolates showed higher transcript levels in the conidia from the 25˚C cultures compared with those from the 37˚C cultures (Fig 6B). Thus, trypacidin production was not correlated to the $tpcC$ transcript level.

In addition to trypacidin, we determined the temperature-dependent regulation of DHN-melanin accumulation in clinical strains. Among these strains, IFM 59056, IFM 59359, IFM 59073, IFM 61407, and IFM 61610, showed deeper conidial pigmentation levels in the 25˚C cultures than in the 37˚C cultures (Fig 7A). In contrast, the IFM 59365 conidia from the 37˚C culture exhibited a darker pigmentation compared with those from the 25˚C culture, whereas no clear difference in pigmentation was seen in the conidia of IFM 60237 and IFM 60514 (Fig 7A). IFM 59365 accumulated both trypacidin and pigments in the conidia from the 37˚C culture, which is suggestive of a link between the temperature-responsive mechanism regulating trypacidin and pigment production in $A. fumigatus$ conidia. An analysis of the $pksP$ transcription level revealed that all of the clinical strains, except IFM 60237 and IFM 61407, exhibited a higher level of the $pksP$ transcript in the conidia from the 25˚C cultures (Fig 7B). The experiments using clinical isolates showed that trypacidin and pigment production vary from strain to strain. Despite the strain-dependent temperature effects on trypacidin and pigment production, the effect on trehalose accumulation in the clinical isolates was largely similar to that observed in Af293, where the conidia from the 37˚C culture contained more trehalose (Fig 7C).
Fig 6. Quantification of trypacidin production in clinical isolates. (A) Samples were extracted from the conidia of clinical isolates as well as Af293 strains that were harvested from 25˚C and 37˚C cultures, and they were analyzed by HPLC. The amount of trypacidin was quantified using a standard curve. Each sample was tested in 3 to 5 replicates. Error bars represent the standard deviation. Significant differences (by Student’s t-test) between conidia from 25˚C and 37˚C cultures were indicated with asterisk (*: p<0.05). (B) Gene expression analysis of tpcC in the clinical isolates. The eight clinical isolates were grown on PDA for 7 d at 25
and 37°C. Then, conidia were harvested, and their RNA was extracted. The expression ratio of tpcc relative to actin was determined by qRT-PCR analysis. Error bars represent standard deviations based on three biological replicates.

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**Discussion**

In general, melanin contributes to the survival of fungal propagules in harsh environmental conditions [29]. Our results support the role of DHN-melanin in protection against UV irradiation in *A. fumigatus* conidia. The conidia from the 25°C culture were sensitive to heat and oxidative stress most probably because of a decreased trehalose content. One possible reason for this reduction is the increased intracellular trehalase activity. TreB is a neutral trehalase

![Figure 7](https://doi.org/10.1371/journal.pone.0177050.g007)

**Fig 7. Effect of temperature on pigmentation in the clinical isolates.** (A) Comparison of conidial pigmentation. Conidia of the clinical isolates were harvested from 25 and 37°C cultures. Conidial suspensions were diluted to 10^8 conidia mL⁻¹ and photographed. (B) Expression analysis of pksP. RNA was extracted from conidia that were produced on PDA at 25 and 37°C. The expression ratio of pksP was determined by qRT-PCR. Each sample was tested in three biological replicates. Error bars represent standard deviations. (C) Trehalose was extracted from the conidia of clinical isolates and quantified by glucose assay after trehalase digestion as described previously [5]. Error bars represent the standard deviation based on three independent replicates. Significant differences (by Student's t-test) between conidia from 25°C and 37°C cultures were indicated with asterisk (**: p<0.01).
involved in trehalose breakdown during the germination stage [30], and the transcript level was relatively high in the conidia harvested from the 25˚C culture (Fig 2B). Importantly, the temperature effects on conidial properties, such as pigmentation, trehalose accumulation, and heat stress tolerance, were also observed in the conidia harvested from cultures on glucose minimal medium (S1 Fig). Collectively, our results revealed that temperature has a great impact on the stress-resistance properties of conidia, which affects their survival in the environment.

DHN-melanin plays an important role in preventing the predation of *A. fumigatus* conidia by soil amoebae [14,31]. Trypacidin also affects the interaction between *A. fumigatus* conidia and amoebae [27]. Indeed, *tpcC* deletion mutant conidia were more sensitive to an amoebae challenge, which suggests that trypacidin is responsible for predation avoidance. Thus, it is tempting to assume that the fungus has evolved a temperature-dependent regulatory mechanism for melanin and trypacidin production to potentiate conidial survival in harsh environmental niches. Further studies will enable us to learn much more about how and why conidia are protected by secondary metabolites.

Trypacidin was initially determined to have an anti/protozoal activity [32]. Trypacidin is cytotoxic to A549 lung cancer cells and human bronchial epithelial cells, indicating that it is a potential mycotoxin [33]. Throckmorton *et al.* [26] showed that the A1163 strain is derived from a clinical isolate and did not produce trypacidin because of the genetic inactivation of the *tpcC* gene, indicating that the production of this mycotoxin is dispensable for the development of aspergillosis in a clinical setting. This view was also supported by virulence tests using a Toll-deficient *Drosophila* model [26], in which there was no significant difference in virulence between a *tpcC* mutant and a WT strain. During infection, conidia must germinate on and in the lung tissues. Thus, it is possible that trypacidin contributes to the injury of attached or surrounding cells, including epithelial cells and phagocytes, during germination. In swelling conidia, there was no increased expression of related genes, such as *tpcC*, *in vitro*, suggesting that there was no *de novo* trypacidin synthesis during the swelling/germinating stage (data not shown). Thus, trypacidin may particularly contribute to conidial protection, and it may not be a prerequisite for *A. fumigatus* virulence.

Our results highlighted the temperature effect on conidial properties, such as stress tolerance, and the accumulation of DHN-melanin and mycotoxin. This should be considered when conidia suspensions are prepared for phenotypic studies, including infection experiments. Different incubation temperatures might affect the results.

Another notable finding is that the isolates exhibited heterogeneous responses, in terms of pigmentation and trypacidin production, to environmental temperature. It is empirically acceptable that all of the isolates of identical species, *i.e.*, *A. fumigatus*, possess a genetically diverse background and, consequently, exhibit different phenotypes, such as hyphal growth, conidiation, conidial pigmentation, nutrient preferences, and virulence [34,35]. The analysis of our set of clinical isolates revealed that some strains produced more densely pigmented conidia in the 25 or 37˚C cultures, whereas others produced highly pigmented conidia equally at both temperatures. Because DHN-melanin protects conidia from UV-irradiation stress, highly pigmented conidia appear to be preferable for conidial survival under UV- or strong sunlight-exposure conditions. The fungus might have evolved the temperature-dependent regulation of stress protection in a strain-dependent manner to adapt to its environment. For a deeper understanding of conidia stress-resistance-related physiology, heterogeneity among isolates should be taken into account, and exploring the diversity of conidial properties may enable the identification of previously unknown stress-resistance factors.

In conclusion, we presented the first evidence that conidial stress resistance and secondary metabolism are affected by temperature during conidiation in *A. fumigatus*. The findings of a low temperature-dependent overproduction of DHN-melanin and trypacidin in conidia...
provide new insights into the conidial physiology of filamentous fungi and pave the way for understanding the temperature-dependent regulation of secondary metabolism.

Material and methods

Strains and growth conditions

*A. fumigatus* strains Af293 was used to generate the ΔtpcC strain. The fungal strains used in this study are listed in S4 Table. The clinical strains IFM 59056, IFM 59359, IFM 60237, IFM 59365, IFM 59073, IFM 60514, IFM 61407, and IFM 61610 were isolated from patients with chronic pulmonary aspergillosis from 2007 to 2012 in Japan, and their detailed descriptions were provided in a previous study [36]. All strains were cultivated in PDA at 25˚C, 37˚C, and 45˚C for conidia collection. The cultivation time was 7 d, unless stated otherwise.

**Construction of the tpcC gene deletion mutant.** The mutants used in this study were constructed by gene replacement method that used a hygromycin B resistant marker (*hygB*) in a double homologous recombination event. The deletion cassettes for each gene were constructed using the GeneArt Seamless Cloning and Assembly Kit (Invitrogen, Carlsbad, CA, USA). To create the deletion cassettes, 5’- and 3’-flanking regions of the target gene and the *hygB* fragment were fused and introduced into the pUC119 vector. The *hygB* fragment was amplified and obtained from pCB1004 by PCR using the primer pair *hygBr*-F and *hygBr*-R (S5 Table). To amplify fragments for cloning, KOD plus ver.2 high-fidelity DNA polymerase (Toyobo, Osaka, Japan) was used for all the PCRs.

To delete *tpcC* (Afu4g14560), 5’- and 3’-flanking regions were obtained by PCR using the primers *tpcC*-U-F (pUC119) and *tpcC*-U-R (*hygBr*), and *tpcC*-D-F (*hygBr*) and *tpcC*-D-R (pUC119) (S5 Table). These flanking regions and the *hygB* fragment were ligated into pUC119 using the GeneArt system, resulting in the plasmid, pUC-*tpcC*:*hygBr*, from which the cassette for transformation was amplified using primers *tpcC*-U-F and *tpcC*-D-R (S5 Table).

Transformation of *A. fumigatus* was performed according to a conventional protoplast-polyethylene glycol transformation method for *Aspergillus* [37]. Homologous recombination and correct gene replacement were confirmed by PCR of genomic DNA, and the absence of the transcript of the target gene was verified by RT-PCR analysis. We obtained at least three independent transformants for each gene deletion, and the data from a representative strain were presented.

**Conidia preparation.** Conidia of each strain were stored in 20% glycerol in a −80˚C freezer. To prepare fresh conidia, the stored conidia were inoculated on a PDA slant and incubated at 37˚C for 1 week. The conidia were harvested with phosphate-buffered saline (PBS) containing 0.1% Tween 20, and the concentration was calculated by counting the conidia with a hemocytometer (Watson, Kobe, Japan). To collect conidia for RNA purification and trypacidin extraction, conidia were mixed with 15 mL of PDA (final concentration, approximately 10⁴ conidia/mL) before the medium solidified in a 100-mL flask, and they were incubated at 25˚C, 37˚C, or 45˚C, for 7 d (or culture age as indicated) in the dark. After the cultivation, conidia were harvested with PBS-Tween 20, filtered by a Miracloth, counted with a hemocytometer, and washed once with PBS-Tween 20. Microscopy observations revealed that there were no hyphal fragment contaminants in the conidial suspension, which contained exclusively conidia.

**Stress test for conidia.** Viability assays for heat stress and oxidative stress were performed as described previously, with slight modifications [5]. Briefly, suspensions of 10⁵ conidia mL⁻¹ were incubated at 60˚C for 15 min (heat stress assay) or in the presence of 200 mM H₂O₂ for 15 min at room temperature (oxidative stress assay). After stress treatment, the suspensions were immediately cooled on ice and diluted to 10³ conidia mL⁻¹. To count colony-forming
units (CFU), the suspensions (100 μL) were spread on 0.1% yeast extract-containing glucose minimal medium plates. Conidia without stress treatment were used as a reference, and survival rates were calculated in three or four technical replicates. CFUs were counted from the plates in each test.

For the UV exposure test, 100 conidia were spread onto 0.1% yeast extract-containing glucose minimal medium plates, and the plates were exposed to 9.55 W m\(^{-2}\) of UV light for 1 min, providing a dose of 573 J m\(^{-2}\). After UV exposure, the plates were incubated at 37°C in the dark for more than 24 h before counting the CFU. The survival rate was calculated by dividing the CFU obtained following UV exposure by the CFU obtained in the absence of UV exposure. CFUs were counted from five plates.

**Polyols and trehalose quantification.** Polyols and trehalose contents in conidia were measured as described previously [5]. Briefly, conidia harvested from each culture condition were suspended in H\(_2\)O and incubated at 98°C for 3 h to extract the polyols and trehalose. After filtration with an Amicon Ultra concentrator (Millipore, Billerica, MA, USA), the contents were quantified by high-performance liquid chromatography using a Shodex Asahipack NH2P-50 4E column. The mean value was obtained from six biological replicates. Trehalose content was also determined by glucose quantification after degrading by trehalase as described previously [5].

**Melanin test.** To compare conidial pigmentations, suspensions of freshly harvested conidia were prepared and diluted to 10\(^8\) conidia mL\(^{-1}\). The pigmentation levels of the conidial suspensions were visually compared in a cuvette. TCZ was used to inhibit the DHN-melanin synthesis pathway [24]. TCZ was added to PDA at a final concentration of 10 μg mL\(^{-1}\). Preliminary experiments confirmed that 1 μg mL\(^{-1}\) was insufficient to repress the production of DHN-melanin, and that 10 μg mL\(^{-1}\) of TCZ did not affect colony growth or conidial production levels.

**RNA and cDNA preparation.** Mycelia and conidia were harvested, washed with distilled water, and frozen in liquid nitrogen, and total RNA was isolated using the FastRNA Pro Red Kit (MP Biomedicals, Santa Ana, CA, USA). For quantitative real-time RT-PCR analysis, to obtain cDNA pools from the total RNA, removal of possible contaminating genomic DNA and reverse transcription were performed using the ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo).

**RNA sequencing analysis.** For RNA-sequencing analysis, sample libraries were prepared from total RNA by the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA) according to standard protocols and as performed previously [5]. Briefly, each total RNA sample (1 μg) was enriched for mRNA using oligo (dT)-tagged beads. The mRNA samples were fragmented into smaller pieces and used to synthesize cDNAs. The library construction involved end repair, A-tailing, adapter ligation, and amplification. The mean length for each library was approximately 280 bp. Sequencing was performed in a single-end 50 base pair mode on a Miseq system (Illumina).

To compare the expression levels of each gene, the sequences were analyzed using a CLC Genomics Workbench (CLC Bio, Aarhus, Denmark). The sequence reads were trimmed and the quality was checked. Only reads with quality values higher than Q30 (approximately 97.2% of the total reads) were used for mapping. The *A. fumigatus* Af293 genome data, which was retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA14003), was used as the template for mapping. From the mapping data, FPKM values were calculated using a program in the CLC Genomics Workbench. The RNA-sequencing read data was deposited to the DDBJ Sequence Read Archive (http://trace.ddbj.nig.ac.jp/dra/index_e.html) under accession No. PRJDB5526.
**MIDDAS-M analysis.** The gene clusters whose transcript levels are coordinately changed in the conidia cultured at 25˚C or 45˚C compared to those at 37˚C were investigated using the MIDDAS-M algorithm [28]. Briefly, the induction ratio of genes at 25˚C or 45˚C over 37˚C were evaluated in the logarithmic form with the base of 2 using the transcriptome data. After the Z-score normalization, the gene cluster expression score were calculated for each gene using the algorithm. The maximum cluster size was set as 30. By setting the threshold of the score as 0.05, candidate clusters were detected. The threshold values are respectively 8743 and 24358 in the 25˚C/37˚C and 45˚C/37˚C datasets.

**Quantitative real-time RT-PCR.** Real-time RT-PCR was performed using SYBR Green detection as described previously [37]. The primer sets used in this study are listed in S5 Table. The actin gene was used as a normalization reference (internal control), and the relative expression ratio to actin gene was calculated. Each sample was tested in three biological replicates.

**Extraction and chemical analysis.** Conidia (10⁹) from each strain were suspended in 100 µL of PBS and ethyl acetate (1:9) and were homogenized using a MagNA Lyser Green Beads tube (Roche, Basel, Switzerland). After centrifugation, the supernatant, consisting of the organic phase, was collected and dried in vacuo. The crude extract was dissolved in 100 µL of dimethyl sulfoxide (DMSO). Samples were injected into a high-performance liquid chromatography (HPLC) WakoSil-II system with a 3C18 HG column (particle size: 3µm; length: 150mm; internal diameter: 3mm) (Wako Pure Chemical Industries, Osaka, Japan). The column was run using a gradient of 10–100% acetonitrile in water over 45 min at a flow rate of 1 mL min⁻¹ and monitored by a photodiode array detector (SPD-M10A VP, Shimadzu Corp., Kyoto, Japan). To quantify trypacidin production, peak areas detected by the absorbance at 330 nm were obtained in 3 to 5 biological replicates. A trypacidin standard was purchased from AdipoGen (San Diego, CA, USA) and used to create a standard curve.

To identify chemical structures, peaks corresponding to trypacidin and monomethylsulochrin were fractionated and approximately 1 mg was collected. Their structures were identified from the following spectroscopic parameters.

Trypacidin: ¹H NMR (500 MHz, CDCl₃): δ = 2.42 (s, 3H), 3.64 (s, 3H), 3.67 (s, 3H), 3.93 (s, 3H), 5.75 (d, J = 1.1 MHz), 6.35 (s), 6.53 (s), 7.08 (d, J = 1.1 MHz); ¹³C NMR (125 MHz, CDCl₃): δ = 23.4, 53.0, 56.3, 56.9, 84.3, 104.2, 105.6, 105.8, 108.6, 137.3, 138.5, 152.3, 158.6, 163.7, 169.7, 175.0, 185.9, 190.7; HR-ESI-MS: m/z 345.0976 [M+H]⁺, calcd. for C₁₈H₁₇O₇: 345.0974.

Monomethylsulochrin: ¹H NMR (500 MHz, CDCl₃): δ = 2.27 (s, 3H), 3.35 (3H), 3.67 (s, 3H), 3.69 (s, 3H), 6.04 (s), 6.44 (s), 6.60 (d, J = 2.3 MHz), 7.01 (d, J = 2.3 MHz); ¹³C NMR (125 MHz, CDCl₃): δ = 22.7, 52.4, 55.9, 56.5, 103.1, 103.4, 108.0, 110.0, 110.7, 128.5, 128.7, 148.2, 156.3, 157. 4, 161.1, 164.5, 166.3, 199.6; HR-ESI-MS: m/z 369.0948 [M+Na]⁺, calcd. for C₁₈H₁₈O₇Na: 369.0947.

**Supporting information**

S1 Fig. Temperature effects found in the conidia from cultures on glucose minimal media (GMM).
(PPTX)

S1 Table. FPKM list for 8953 genes.
(XLSX)

S2 Table. FPKM list for the allergenic genes.
(XLSX)
S3 Table. FPKM list for the genes that were coordinately regulated.
(XLSX)

S4 Table. Strains used in this study.
(XLSX)

S5 Table. PCR primers used in this study.
(XLSX)

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Author Contributions

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Investigation: DH KS SS MU TN NK.
Project administration: DH.
Supervision: HO AW SK TG KK.
Writing – original draft: DH.

References

1. Wyatt TT, Wösthen HA, Dijkstra JH (2013) Fungal spores for dispersion in space and time. Adv Appl Microbiol 85: 43–91. https://doi.org/10.1016/B978-0-12-407672-3.00002-2 PMID: 23942148

2. Ruijter GJ, Bax M, Patel H, Flinter SJ, van de Vondervoort PJ, de Vries RP, et al. (2003) Mannitol is required for stress tolerance in Aspergillus niger conidiospores. Eukaryot Cell 2: 690–698. https://doi.org/10.1128/EC.2.4.690-698.2003 PMID: 12912888

3. Jørgensen TR, Park J, Arentshorst M, van Welzen AM, Lamers G, Vankuyk PA, et al. (2011) The molecular and genetic basis of conidial pigmentation in Aspergillus niger. Fungal Genet Biol 48: 544–553. https://doi.org/10.1016/j.fgb.2011.01.005 PMID: 21277986

4. Sakamoto K, Iwashita K, Yamada O, Kobayashi K, Mizuno A, Akita O, et al. (2009) Aspergillus oryzae atfA controls conidial germination and stress tolerance. Fungal Genet Biol 46: 887–897. https://doi.org/10.1016/j.fgb.2009.09.004 PMID: 19770065

5. Hagiwara D, Suzuki S, Kamel K, Goni T, Kawamoto S (2014) The role of AtfA and HOG MAPK pathway in stress tolerance in conidia of Aspergillus fumigatus. Fungal Genet Biol 73: 138–149. https://doi.org/10.1016/j.fgb.2014.10.011 PMID: 25499537

6. Hagiwara D, Sakamoto K, Abe K, Gomi K (2016) Signaling pathways for stress responses and adaptation in Aspergillus species: stress biology in the post-genomic era. Biosci Biotechnol Biochem 80: 1667–1680. https://doi.org/10.1080/09168451.2016.1162085 PMID: 27007956

7. Fillinger S, Ruijter G, Tamás MJ, Visser J, Thevelein JM, d’Enfert C (2001) Molecular and physiological characterization of the NAD-dependent glycerol 3-phosphate dehydrogenase in the filamentous fungus Aspergillus nidulans. Mol Microbiol 39: 145–157. PMID: 11123996

8. Osherov N (2012) Interaction of the pathogenic mold Aspergillus fumigatus with lung epithelial cells. Front Microbiol 3: 346. https://doi.org/10.3389/fmicb.2012.00346 PMID: 23055997

9. Kwon-Chung KJ, Sugui JA (2013) Aspergillus fumigatus—what makes the species a ubiquitous human fungal pathogen? PLoS Pathog 9: e1003743. https://doi.org/10.1371/journal.ppat.1003743 PMID: 24348239

10. Heinekamp T, Thywissen A, Macheleidt J, Keller S, Valiante V, Brakhage AA (2013) Aspergillus fumigatus melanins: interference with the host endocytosis pathway and impact on virulence. Front Microbiol 3: 440. https://doi.org/10.3389/fmicb.2012.00440 PMID: 23346079
11. Tsai HF, Chang YC, Washburn RG, Wheeler MH, Kwon-Chung KJ (1998) The developmentally regulated alb1 gene of *Aspergillus fumigatus*: its role in modulation of conidial morphology and virulence. J Bacteriol 180: 3031–3038. PMID: 9620950
12. Volling K, Thywissen A, Brakhage AA, Saluz HP (2011) Phagocytosis of melanized *Aspergillus* conidia by macrophages exerts cytoprotective effects by sustained PI3K/Akt signalling. Cell Microbiol 13: 1130–1148. https://doi.org/10.1111/j.1462-5822.2011.01605.x PMID: 21501368
13. Thywissen A, Heinekamp T, Dahse HM, Schmaler-Ripcke J, Nietzsche S, Zipfel PF, et al. (2011) Conidial Dihydroxy napthalene Melanin of the Human Pathogenic Fungus *Aspergillus fumigatus* Interferes with the Host Endocytosis Pathway. Front Microbiol 2: 96. https://doi.org/10.3389/fmicb.2011.00096 PMID: 21747802
14. Hillmann F, Novohradska S, Mattern DJ, Forberger T, Heinekamp T, Westermann M, et al. (2015) Virulence determinants of the human pathogenic fungus *Aspergillus fumigatus* protect against soil amoeba predation. Environ Microbiol 17: 2858–2869. https://doi.org/10.1111/1462-2920.12808 PMID: 25664622
15. Geib E, Gressler M, Viedemikova I, Hillmann F, Jacobsen ID, Nietzsche S, et al. (2016) A non-canonical melanin biosynthesis pathway protects *Aspergillus terreus* conidia from environmental stress. Cell Chem Biol 23: 587–597. https://doi.org/10.1016/j.chembiol.2016.03.014 PMID: 27133313
16. Low SY, Dannemiller K, Yao M, Yamamoto N, Peccia J (2011) The allergenicity of *Aspergillus fumigatus* conidia is influenced by growth temperature. Fungal Biol 115: 625–632. https://doi.org/10.1016/j.funbio.2011.03.006 PMID: 21724168
17. Misaghi J, Grogan RG, Duniway JM, Kimble KA (1978) Influence of environment and culture media on spore morphology of *Alternaria alternata*. Phytopathology 68: 29–34.
18. Phillips J (1982) Changes in conidia production temperature on germination and infectivity of *Alternaria helianthi*. Phytopathology 85:677–682.
19. Abbas HK (1995) Effect of conidia production temperature on germination and infectivity of *Alternaria helianthi*. Phytopathology 85:677–682.
20. Bertolini P Tian SP (1997) Effect of temperature of production of *Botrytis allii* conidia on their pathogenicity to harvested white onion bulbs. Plant Pathology 46: 432–438.
21. Rangel DE, Braga GU, Fernandes EK, Keyser CA, Hallsworth JE, Roberts DW (2015) Stress tolerance and virulence of insect-pathogenic fungi are determined by environmental conditions during conidial formation. Curr Genet 61: 383–404. https://doi.org/10.1007/s00294-015-0477-y PMID: 25791499
22. Braga GU, Rangel DE, Fernandes EK, Flint SD, Roberts DW (2015) Molecular and physiological effects of environmental UV radiation on fungal conidia. Curr Genet 61: 405–425. https://doi.org/10.1007/s00294-015-0483-0 PMID: 25824285
23. Al-Bader N, Vanier G, Liu H, Gravelat FN, Urb M, Hoareau CM, et al. (2010) Role of trehalose biosynthesis in *Aspergillus fumigatus* development, stress response, and virulence. Infect Immun 78: 3007–3018. https://doi.org/10.1128/IAI.00813-09 PMID: 20439478
24. Piet M, Vandeputte P, Tronchin G, Renier G, Saulnier P, Georgeault S, et al. (2009) Melanin is an essential component for the integrity of the cell wall of *Aspergillus fumigatus* conidia. BMC Microbiol 9: 177 https://doi.org/10.1186/1471-2180-9-177 PMID: 19703288
25. Sanchez JF, Somoza AD, Keller NP, Wang CC (2012) Advances in *Aspergillus* secondary metabolism research in the post-genomic era. Nat Prod Rep 29: 351–371. https://doi.org/10.1039/c2np00084a PMID: 22283666
26. Throckmorton K, Lim FY, Kontoyiannis DP, Zheng W, Keller NP (2015) Redundant synthesis of a conidial polypeptide by two distinct secondary metabolite clusters in *Aspergillus fumigatus*. Environ Microbiol 18: 246–259. https://doi.org/10.1111/1462-2920.13007 PMID: 26242966
27. Mattern DJ, Schoeler H, Weber J, Novohradska S, Kraiboon K, Dahse HM, et al. (2015) Identification of the antiphagocytic trepacidin gene cluster in the human-pathogenic fungus *Aspergillus fumigatus*. Appl Microbiol Biotechnol 99: 10151–10161. https://doi.org/10.1007/s00253-015-6898-1 PMID: 26278536
28. Umemura M, Koike H, Nagano N, Ishii T, Kawano J, Yamane N, et al. (2013) MIDDAS-M: motif-independent de novo detection of secondary metabolite gene clusters through the integration of genome sequencing and transcriptome data. PLoS One 8: e840128.
29. Eisenman HC, Casadevall A (2012) Synthesis and assembly of fungal melanin. Appl Microbiol Biotechnol 93: 931–940. https://doi.org/10.1007/s00253-011-3777-2 PMID: 22173481
30. d’Enfert C, Bonini BM, Zapella PD, Fontaine T, da Silva AM, Terenzi HF (1999) Neutral trehalases catalyse intracellular trehalose breakdown in the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. Mol Microbiol 32: 471–483. PMID: 10320571
31. Van Waeyenbergh L, Bare J, Pasmans F, Claesys M, Bert W, Haesebrouck F, et al. (2013) Interaction of *Aspergillus fumigatus* conidia with *Acanthamoeba castellanii* parallels macrophage-fungus interactions. Environ Microbiol Rep 5: 819–824. https://doi.org/10.1111/1758-2229.12082 PMID: 24249290

32. Balan J, Ebringer L, Nemec P, Kovac S, Dobias J (1963) Antiprotozoal antibiotics. II. Isolation and characterization of trypacidin, a new antibiotic, active against *Trypanosoma cruzi* and *Toxoplasma gondii*. J Antibiot (Tokyo) 16: 157–160.

33. Gauthier T, Wang X, Sifuentes Dos Santos J, Fysikopoulos A, Tadrist S, et al. (2012) Trypacidin, a spore-borne toxin from *Aspergillus fumigatus*, is cytotoxic to lung cells. PLoS One 7: e29906. https://doi.org/10.1371/journal.pone.0029906 PMID: 22319557

34. Kowalski CH, Beattle SR, Fuller KK, McGurk EA, Tang YW, Hohl TM, et al. (2016) Heterogeneity among isolates reveals that fitness in low oxygen correlates with *Aspergillus fumigatus* virulence. MBio 7: e01515–16.

35. Fuller KK, Cramer RA, Zegans ME, Dunlap JC, Loros JJ (2016) *Aspergillus fumigatus* photobiology illuminates the marked heterogeneity between isolates. MBio 7: e01517–16.

36. Takahashi-Nakaguchi A, Muraosa Y, Hagiwara D, Sakai K, Toyotome T, Watanabe A, et al. (2015) Genome sequence comparison of *Aspergillus fumigatus* strains isolated from patients with pulmonary aspergillosis and chronic necrotizing pulmonary aspergillosis. Med Mycol 53: 353–360. https://doi.org/10.1093/mmy/myv003 PMID: 25851262

37. Hagiwara D, Takahashi-Nakaguchi A, Toyotome T, Yoshimi A, Abe K, Kamei K, et al. (2013) NikA/TcsC histidine kinase is involved in conidiation, hyphal morphology, and responses to osmotic stress and antifungal chemicals in *Aspergillus fumigatus*. PLoS One 8: e80881. https://doi.org/10.1371/journal.pone.0080881 PMID: 24312504