An opportunistic human pathogen on the fly: Strains of *Aspergillus flavus* vary in virulence in *Drosophila melanogaster*

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**Abstract**

Aspergilloses are fungal diseases in humans and animals that is caused by members of the genus *Aspergillus*. *Aspergillus flavus* is an important opportunistic pathogen, second only to *A. fumigatus* as a cause of human aspergillosis. Differences in virulence among *A. flavus* isolates from clinical and other substrates and mating types are not well known. The fruit fly *Drosophila melanogaster* has become a model organism for investigating virulence of human pathogens due to similarities between its immune system and that of mammals. In this study we used *D. melanogaster* as a model host to compare virulence among *A. flavus* strains obtained from clinical sources as compared with other substrates, between isolates of different mating types, and between isolates of *A. flavus* and *A. fumigatus*. Anesthetized flies were infected with *A. flavus*; mortality ranged from 15% to >90%. All strains were virulent, but some were significantly more so than others, which in turn led to the wide mortality range. Clinical strains were significantly less virulent than environmental strains, probably because the clinical strains were from culture collections and the environmental strains were recent isolates. Mean virulence did not differ between MAT1–1 and MAT1–2 mating types and the phylogeny of *A. flavus* isolates did not predict virulence. *A. flavus* was on average significantly more virulent than *A. fumigatus* on two lines of wild-type flies, Canton-S and Oregon-R. *D. melanogaster* is an attractive model to test pathogenicity and could be useful for identifying genes involved in virulence.

**Key words:** Aspergillosis, clinical strain, emerging pathogen, substrate, mating type.

**Introduction**

Aspergilloses are diseases caused by members of the genus *Aspergillus* that affect humans and a broad spectrum of other mammals [1,2]. In the United States, human aspergillosis has a prevalence of roughly 1 in 100 000, which translates to about seven people becoming infected every day [3]. Aspergillosis patients spend an average of $95,000 on treatment, making it the most expensive fungal disease per patient [4]. Aspergillosis can cause aspergillomas, which
are infections of different tissues, and invasive pulmonary disease [5].

A. fumigatus is the species most commonly involved in human aspergillosis [6, 7]. However, A. flavus is an emerging opportunistic pathogen that is responsible for approximately 30% of all aspergillosis cases in the United States and 50%–80% of Aspergillus-associated sinusitis, keratitis, and cutaneous infections in the Middle East, Africa, and Southeast Asia [2, 5, 8]. Its ubiquity in nature, ease of dispersal, and small conidia (2–5 µm) make it very difficult to limit exposure of all potential hosts. The main portal of entry is the respiratory system where inhaled spores are found in the distal areas of the lungs [9]. Infection depends on immunocompromised status of the individual, the degree of exposure, and the virulence of the fungus [10]. However, the relative importance of these three factors is not known, and little attention has been given to exploring differences in virulence among strains.

**Sexuality of A. flavus**

Mating type idiomorphs (MAT1-1 and MAT1-2) were recently discovered in A. flavus, which was long believed to be asexual [11]. Mating types are found both locally and globally in 1:1 proportion, suggesting that recombination occurs in natural populations [11, 12]. However, a high proportion of clinical strains were MAT1-1 (85%), suggesting that a gene linked to the MAT1-1 idiomorph could play a role in virulence [12]. Similarly, the proportion of MAT1-1 to MAT1-2 genotypes in A. fumigatus is 4:1 among isolates from cases of invasive aspergillosis. However, the mating type ratio is 1:1 among noninvasive strains obtained from other substrates, suggesting the potential association between the MAT1-1 mating type and invasiveness [13]. Moreover, larvae of the wax moth Galleria mellonella had lower survival when inoculated with MAT1–1 strains of A. fumigatus than with MAT1-2 [14]. These studies suggest that mating type in Aspergillus could be associated with virulence or ability to infect animal hosts.

**Drosophila melanogaster as a model for aspergillosis**

Because experimental inoculation of humans is not feasible, mice are currently the standard model for aspergillosis studies [15, 16]. Mouse models have been used to explore fungal virulence factors and host factors involved in susceptibility and to evaluate antifungal agents and/or immunomodulatory agents for use as treatments [16]. Recently, the fruit fly D. melanogaster has emerged as a powerful and innovative alternative for pathogenicity studies [17–21]. Its immune system shares traits with the mammalian innate immune system, making it an attractive model for studying host–pathogen interactions [22, 23].

At present, it is unclear whether A. flavus strains vary in their virulence for animals and how this variability could affect their hosts. In this study, a wide range of A. flavus strains from different substrates and of both mating types were tested for pathogenicity on D. melanogaster in order to answer the following questions: (1) Are there differences in virulence among A. flavus strains from different substrates? We hypothesize that A. flavus strains will differ in virulence because adaptations to particular substrates affect the ability to attack animal hosts. (2) Are clinical strains more virulent than environmental strains? We predict that clinical strains will cause higher mortality in flies due to the adaptations mentioned above, and their ability to attack the hosts. (3) Does virulence in A. flavus differ between mating types? Because it has been suggested that genes linked to the mating idiomorphs could play a role in virulence [12], we hypothesize that A. flavus mating types will differ in virulence on D. melanogaster. (4) Is A. flavus more virulent than A. fumigatus in Drosophila? Because A. flavus is known to parasitize insects and A. fumigatus is not, we predicted that A. flavus would be more virulent.

**Materials and methods**

**Drosophila maintenance**

Female flies were used in all experiments to minimize sex-dependent effects on susceptibility to infection [24]. Wild-type (WT) Canton-S flies were used instead of the Toll-deficient mutant flies used in previous studies [17, 25] because WT flies are less laborious to manipulate, permitting an increase in sample size. Furthermore, A. flavus strains were sufficiently virulent to colonize WT flies (see below).

Flies were grown on Nutri-fly medium (Genesee Scientific, San Diego, CA, USA), solidified with 10 g/l of agar (Difco), and supplemented with 1 M propionic acid and 0.1% Tegosept as preservative and antifungal agent, respectively [24]. While flies were cultured at approximately 25°C in natural light, incubation conditions were altered after infection with A. flavus, such that the flies were incubated at a temperature of 28°C (±1°C) and with constant light, conditions that favor growth of the pathogen. Additionally, constant light weakens the flies’ immune system by disrupting circadian rhythms, increasing the hosts’ susceptibility to infection [26].

**Aspergillus flavus strains**

Thirty-one A. flavus strains whose phylogenetic relationships and mating types were determined previously were
Table 1. *Aspergillus flavus* and *A. fumigatus* strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mating type</th>
<th>Substrate</th>
<th>Host</th>
<th>Clade (mean ± standard deviation)</th>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JO31</td>
<td>MAT1-1</td>
<td>Sea fans</td>
<td>Canton-S</td>
<td>III 4.9 (5.6)</td>
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<tr>
<td>ABPMA2</td>
<td>MAT1-1</td>
<td>Sea fans</td>
<td>Canton-S</td>
<td>III 4.6 (5.8)</td>
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<td>MAT1-1</td>
<td>Sea fans</td>
<td>Canton-S</td>
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</tr>
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<td>MAT1-1</td>
<td>Sea fans</td>
<td>Canton-S</td>
<td>III 2.9 (2.9)</td>
</tr>
<tr>
<td>JO27</td>
<td>MAT1-1</td>
<td>Sea fans</td>
<td>Canton-S</td>
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<tr>
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<td>Marine algae</td>
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<tr>
<td>70stockwrt</td>
<td>MAT1-1</td>
<td>Soil</td>
<td>Canton-S</td>
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<tr>
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<td>MAT1-1</td>
<td>Air</td>
<td>Canton-S</td>
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<td>MAT1-1</td>
<td>Coffee beans</td>
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<tr>
<td>30M</td>
<td>MAT1-1</td>
<td>Clinical</td>
<td>Canton-S</td>
<td>IV 7.2 (5.9)</td>
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<tr>
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<td>MAT1-1</td>
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<td>Canton-S</td>
<td>V 9.8 (12.5)</td>
</tr>
<tr>
<td>HP1</td>
<td>MAT1-1</td>
<td>Clinical</td>
<td>Canton-S</td>
<td>— 6.6 (7.9)</td>
</tr>
<tr>
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<td>MAT1-1</td>
<td>Clinical</td>
<td>Canton-S</td>
<td>IV 5.7 (7.2)</td>
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<tr>
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<td>Canton-S</td>
<td>IV 11 (6.9)</td>
</tr>
<tr>
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<td>MAT1-1</td>
<td>Clinical</td>
<td>Canton-S</td>
<td>V 15 (11.5)</td>
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<tr>
<td>ATCC24133</td>
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<td>Clinical</td>
<td>Canton-S</td>
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<tr>
<td>250M</td>
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<td>Canton-S</td>
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<tr>
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<td>MAT1-2</td>
<td>Sea fans</td>
<td>Canton-S</td>
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<td>Canton-S</td>
<td>II 3 (3.4)</td>
</tr>
<tr>
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<td>MAT1-2</td>
<td>Air</td>
<td>Canton-S</td>
<td>I 0.9 (1.7)</td>
</tr>
<tr>
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<td>Air</td>
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</tr>
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<td>Sea fans</td>
<td>Canton-S</td>
<td>— 3.2 (4)</td>
</tr>
<tr>
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<td>Airborne</td>
<td>Canton-S</td>
<td>I 5.8 (4.9)</td>
</tr>
<tr>
<td>SAF25</td>
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<td>Soil</td>
<td>Canton-S</td>
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<td>MAT1-2</td>
<td>Sea fans</td>
<td>Canton-S</td>
<td>IV 9.6 (5)</td>
</tr>
<tr>
<td>AB10</td>
<td>MAT1-2</td>
<td>Air</td>
<td>Canton-S</td>
<td>V 7.9 (7.6)</td>
</tr>
<tr>
<td>CB35</td>
<td>MAT1-2</td>
<td>Sea fans</td>
<td>Canton-S</td>
<td>— 6.6 (9.2)</td>
</tr>
<tr>
<td>CA14N1</td>
<td>MAT1-2</td>
<td>Pistachio bud</td>
<td>Canton-S</td>
<td>— 2.8 (2.9)</td>
</tr>
<tr>
<td>olgA</td>
<td>MAT1-2</td>
<td>Pistachio bud</td>
<td>Canton-S</td>
<td>— 3.2 (1.5)</td>
</tr>
<tr>
<td>MYA873</td>
<td>MAT1-2</td>
<td>Clinical</td>
<td>Canton-S</td>
<td>— 29.6 (3.2)</td>
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<table>
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<th>Aspergillus fumigatus</th>
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<th>Substrate</th>
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<th>Clade (mean ± standard deviation)</th>
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<td>636M</td>
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<td>Clinical</td>
<td>Canton-S</td>
<td>— 28 (4.8)</td>
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<td>CAI</td>
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<td>Sawdust compost</td>
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<td>Clinical</td>
<td>Oregon-R</td>
<td>— 34.1 (1.3)</td>
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<tr>
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<td>Unknown</td>
<td>Clinical</td>
<td>Oregon-R</td>
<td>— 34.7 (0.7)</td>
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<td>CAI</td>
<td>Unknown</td>
<td>Sawdust compost</td>
<td></td>
<td>Oregon-R</td>
</tr>
</tbody>
</table>

Dashes represent *Aspergillus* strains without bootstrap support or not placed in a phylogenetic tree [12].

tested for their virulence (Table 1) [12]. To produce conidia for fly inoculation, *A. flavus* strains were grown on yeast agar glucose (YAG) medium supplemented with 0.01 M magnesium sulfate and incubated at 37°C for 3–4 days [24]. To compare results obtained in previous studies with those for *A. fumigatus*, three *A. fumigatus* isolates were included in the present study as a form of control (Table 1). To compare results obtained with the Canton-S line of *D. melanogaster* to those from previous studies using the Oregon-R line, both lines were infected with one *A. flavus* strain and three *A. fumigatus* strains.

**Infection assay**

More than 10 000 flies were infected in rolling assays by shaking flies in Petri dishes containing sporulating cultures of *A. flavus* as previously described [17, 24]. We chose this method over others (e.g., injection) because it is fast, easy,
and causes minimal injury. Female flies, aged 3–4 days, in tubes with 35 (±1) flies, were anesthetized with carbon dioxide and placed on a plate containing sporulating colonies of A. flavus growing on YAG medium. The plates were then agitated for approximately 1 min. As a control, flies were agitated on a YAG plate without fungi. Inoculum was quantified and standardized by putting individual flies in 200 µl of sterile water with 0.01% Tween 80, vortexing to remove conidia, and counting conidia in a hemocytometer. Inoculated flies had 1–4 × 10^5 conidia per fly on their bodies; we did not determine the concentration of conidia colonizing the internal tissues. After inoculation, flies were incubated at approximately 28°C and monitored daily as mentioned above. Each strain of A. flavus was used to inoculate three tubes of flies per inoculation date, and all experiments were replicated three times on different dates. Mortality was recorded daily for 8 days.

To confirm that dead flies were infected with A. flavus, the flies were rinsed three times in sterile water with 0.01% Tween 80 to remove conidia, surface-sterilized in 0.5% sodium hypochlorite (Clorox 10%) for 3 min, and rinsed three times with sterile water. Surface-sterilized flies were put on (a modification of [27]) or water agar and observed for growth of A. flavus (Fig. 1). Surviving flies were also disinfected and plated after 8 days to confirm that inoculation with A. flavus was successful.

**Statistical analysis**

The survival of infected flies (i.e., number of flies alive per tube, from 0 to 35) was used as a measure of fungal virulence. It was calculated using Kaplan–Meier analysis in JMP, version 8, for Mac, with number of days survived as the time to event; flies still alive on day 8 after infection were included as censored observations [14]. Differences in virulence among strains of A. flavus were compared using analysis of variance (ANOVA) followed by Tukey–Kramer honestly significant difference (HSD) tests to find which means were significantly different from one another. The same analyses were used to test differences in virulence between A. flavus and A. fumigatus and between Canton-S and Oregon-R WT flies.

To test for a relationship between virulence and phylogeny within A. flavus, a maximum parsimony tree was generated with PAP*, version 4.0b 10 [28], based on amplified fragment length polymorphism (AFLP) data from a previous study [12]. Virulence was compared among clades with bootstrap values >75% using ANOVA and Tukey–Kramer HSD in JMP, version 8.

**Results**

**Host–pathogen interactions**

All strains caused infection and mortality in female flies (Fig. 2A), with mortality ranging from 15% to >90%. Uninfected control flies had 1%–10% mortality, probably due to manipulations of the rolling assay. Infection by A. flavus was 100% in exposed flies and 0% in control flies, proving that the rolling method of inoculation was effective.

Virulence differed significantly between infected strains and uninoculated controls and among fungal strains (Tukey–Kramer HSD; P < 0.0001; Fig. 2A). Strain ABPMA1 (isolated from air in Chitré, Herrera, Panama) was the most virulent, causing >90% mortality by day 8, while the clinical strain MYA873 (from American Type Culture Collection [ATCC], originally isolated from an aortic valve prosthesis in Spain) had the lowest virulence with 15% mortality (Fig. 2A). In most cases, substrate from which the isolate was obtained did not predict virulence, as A. flavus strains from sea fans, marine algae, air, soil, pistachio bud, and coffee beans were found to have approximately the same virulence (0.12 < P > 1.0; Fig. 2B). However, clinical strains were significantly less virulent than pooled environmental strains, except for those from coffee beans (P < 0.0001; Fig. 2C).

A. flavus was significantly more virulent than A. fumigatus in both Canton-S and Oregon-R WT flies (P < 0.0001; Fig. 3), while A. fumigatus was significantly more virulent in Canton-S than in Oregon-R WT flies. However,

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**Figure 1.** Aspergillus flavus sporulating on a dead wild-type Drosophila melanogaster. All flies that died were plated to ensure the inoculation was successful.
Figure 2. Virulence of *Aspergillus flavus* strains measured by and inversely proportional to survival of *Drosophila melanogaster* after infection. (A) *A. flavus* strains showing low, intermediate, and high levels of virulence; (B) strains grouped by substrate (clinical, sea fans, marine algae, air, soil, pistachio bud, and coffee beans); and (C) clinical vs. environmental strains and mating types MAT1–1 vs. MAT1–2. The y-axis shows proportion of flies surviving.

Differences in virulence of both fungi (combined) on Canton-S vs. Oregon-R flies were not significant (*P* < 0.10; data not shown).

When compared among clades of *A. flavus* as defined by AFLP markers, virulence differed significantly between clades I–III and clades IV–V (*P* < 0.0001; Fig. 4). However, this difference may be a result of unequal distribution of the less virulent clinical strains among clades. Clades I–III included only environmental strains and were more virulent than clades IV and V, which were composed primarily of clinical strains (except ESC7L, ATCC24133, and AB10). Apart from this difference, phylogeny did not predict virulence.

**Virulence and mating type**

Overall, no significant difference in virulence was found between strains of MAT1–1 and MAT1–2 mating types (*P* > 0.2457). However, among clinical strains, MAT1–1 strains were significantly more virulent than those of the single MAT1–2 strain tested (Fig. 2C).

**Discussion**

Differences in virulence between strains of *A. flavus* from clinical and environmental substrates

In general, strains of *A. flavus* isolated from a variety of environmental substrates were virulent in *Drosophila*, and the
extent of virulence was approximately the same regardless of the substratum from which the isolate was obtained. This suggests that any strain of *A. flavus* can colonize *Drosophila* and, by extrapolation, may be able to colonize immunocompromised humans [12,29]. This is an alarming conclusion, given that *A. flavus* is among the world’s most common eukaryotes [1].

Clinical strains were significantly less virulent in *Drosophila* than those recovered from environmental substrates (combined; Fig. 2B, 2C). A study involving the infection of *G. mellonella* larvae with several *A. fumigatus* strains showed that clinical isolates were less virulent than those from environmental sources [14].
The lower virulence of clinical strains may be due, at least in part, to repeated subculturing or length of time in culture [14]. Several of the clinical strains came from the ATCC, and some had been stored in culture for decades. In contrast, most of the environmental strains were only recently collected from the indicated sources. This pattern of loss of virulence in culture is very common in fungi [30]. For instance, it was shown that *Metarhizium anisopliae* was less virulent against *Helicoverpa armigera* after 20–40 serial subcultures [31]. Similarly, decline in virulence and changes in the spore surface properties were seen after repeated subculturing in *M. anisopliae* [32]. *A. flavus* often degenerates when serially transferred on culture media in the laboratory, resulting in phenotypic changes and loss of aflatoxin production [33]. These results may be due to the fact that virulence and other characteristics are under continual natural selection in the environment as a result of competition with other microorganisms and exposure to suboptimal conditions for growth, whereas in pure culture there is less selective pressure for these traits [34].

Sex and virulence: Mating types of *A. flavus*

Among clinical strains, MAT1–1 isolates were significantly more virulent, but it should not be forgotten that only one MAT1–2 strain was investigated (Fig. 2C). Unfortunately, no other clinical MAT1–2 strains were available to test the validity of this result; therefore, it must remain questionable at the present time. Previously we showed that a collection of clinical strains was significantly skewed toward MAT1–1 [11,12], suggesting that genes associated with or linked to mating type idiomorphs could play a role in pathogenicity [12]. However, no difference in virulence between mating types of *A. flavus* was seen when all strains were considered.

Previous studies have shown an association between mating type and virulence in *Aspergillus* species. *A. fumigatus* strains of invasive origin were more commonly MAT1–1 mating type and had higher pathogenicity (as estimated by the elastase activity index) than environmental strains [13]. On the other hand, a study of *A. fumigatus* infection of embryonated chicken eggs suggested that both mating types had similar virulence on this substratum [35]. The association of virulence with mating types in *Aspergillus* warrants further work and may eventually suggest novel strategies for disease prevention and treatment.

**Virulence in *A. flavus* vs. *A. fumigatus* in two *Drosophila* lines**

Previous studies of fungal virulence in *Drosophila* used Toll-deficient mutant flies because most fungi (except *Mucorales, Fusarium moniliforme*, and *Candida albicans*) show limited ability to attack WT flies [36–38]. Here we demonstrate that most strains of *A. flavus* are highly virulent on WT flies and significantly more virulent than the three *A. fumigatus* isolates included in this investigation (Fig. 3). This higher virulence is not a result of the use of Canton-S flies in place of the more commonly studied Oregon-R [17,23,29]. Rather we found that the virulence of *A. flavus* did not differ significantly on Canton-S vs. Oregon-RWT flies (Fig. 3). The remarkable virulence of *A. flavus* may reflect its capacity to attack a wide range of hosts in nature, including insects [29,39]. The virulence of *A. flavus* on WT flies shown here is also higher than that of *A. fumigatus* inoculated on Toll-deficient flies using the same rolling assay [17].

*A. fumigatus* was slightly more virulent on Canton-S than on Oregon-R WT flies, which may be the results of different susceptibility of the inbred WT flies. The same has been documented in inbred mice of various genetic backgrounds against the AF293 strain (used in this investigation) [40].

The Toll pathway regulates innate immune responses in *Drosophila* and is used as defense against fungal infection [41]. Apparently, the Toll pathway is less effective in defending attack by *A. flavus*, which we show here to be the first known *Aspergillus* species to cause aspergillosis in WT flies. It will be important to determine whether the Toll pathway plays any role in defense against *A. flavus* by experiments with Toll-deficient flies.

**Virulence factors**

*A. flavus* strains varied significantly in virulence, and this variability is most likely associated with virulence factors. This explanation was suggested for differences in virulence among strains of *A. fumigatus* and *A. terreus* in Toll-deficient *Drosophila* flies during invasive aspergillosis [25]. Virulence factors for *Aspergillus* on animals are not well characterized [10]; however, in insects it is thought that proteases are necessary for infection [29]. In contrast, several virulence factors have been documented for *Aspergillus* infections of plants. For instance, *A. flavus* L type strains (large sclerota; >400 µm in diameter) were more successful and aggressive on colonizing cotton boll locules than S type strains (small sclerota; <400 µm in diameter) [42]. This difference in virulence was attributed to production of a specific pectinase isoenzyme (Pec2) that facilitates the spread between cotton boll locules [43,44].

Our study included only one S type strain (#70, from cotton field soil in Arizona [42]), which was found to be among the most virulent isolates tested. However, all clinical strains used in this study were L type, so the L type is obviously able to infect mammalian hosts. The data show...
that if virulence factors are involved in infections of humans by *A. flavus*, they are equally common in strains from different substrates and intraspecific clades. This is logical given that humans are presumably dead-end hosts for *A. flavus*, so there is no opportunity for natural selection to favor virulence directly.

**Relationship between phylogeny and virulence**

Although some clades of *A. flavus* were significantly more virulent than others (Fig. 4), the difference may reflect higher proportions of clinical strains (lower in virulence) in some clades than in others, as phylogenetic position did not appear to predict virulence. A similar conclusion was found for the rice blast fungus *Pyricularia oryzae* isolated from the Philippines where multiple pathotypes (virulence patterns) were seen in each of the six lineages detected [45]. Similarly, a phylogeographic study of the citrus brown spot pathogen, *Alternaria alternata*, produced three phylogenetic lineages with differences in the virulence of test isolates distributed among clades [46]. Virulence in fungi is complex, as these studies have shown. This complexity makes *Drosophila* an excellent model because many strains, mutants, and conditions can be tested efficiently in terms of time, space, and resources.

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**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

**References**


