Lack of Host Specialization in *Aspergillus flavus*

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***Aspergillus* spp. cause disease in a broad range of organisms, but it is unknown if strains are specialized for particular hosts. We evaluated isolates of *Aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus nidulans* for their ability to infect bean leaves, corn kernels, and insects (*Galleria mellonella*). Strains of *A. flavus* did not affect nonwounded bean leaves, corn kernels, or insects at 22°C, but they killed insects following hemocoelic challenge and caused symptoms ranging from moderate to severe in corn kernels and bean leaves injured during inoculation. The pectinase P2c, implicated in aggressive colonization of cotton bolls, is produced by most *A. flavus* isolates, but its absence did not prevent colonization of bean leaves. Proteases have been implicated in colonization of animal hosts. All *A. flavus* strains produced very similar patterns of protease isozymes when cultured on horse lung polymers. Quantitative differences in protease levels did not correlate with the ability to colonize insects. In contrast to *A. flavus*, strains of *A. nidulans* and *A. fumigatus* could not invade living insect or plant tissues or resist digestion by insect hemocytes. Our results indicate that *A. flavus* has parasitic attributes that are lacking in *A. fumigatus* and *A. nidulans* but that individual strains of *A. flavus* are not specialized to particular hosts.**

Aspergillus species are associated with disease in plants, insects, man, and other animals (4, 14, 24). For example, *Aspergillus flavus* causes disease of agronomically important crops, such as corn and peanuts, is second only to *Aspergillus fumigatus* as the cause of human invasive aspergillosis, and is the *Aspergillus* species most frequently reported to infect insects.

Aspergillus spp. are generally regarded as opportunistic pathogens that require wounds or otherwise weakened hosts for colonization (24). *A. flavus*, however, also has limited parasitic abilities and, in some cases, can directly invade seeds and colonize living tissues (20). We found previously (35) that *Aspergillus* spp. produced a much broader spectrum of protein and polysaccharide-hydrolyzing enzymes than did specialized pathogens, which may be indicative of their less-specialized lifestyle. Shieh et al. (29), however, found that aggressiveness toward cotton depended on production of a specific pectinase isoenzyme (P2c) that facilitates spread between cotton boll locules. Weakly pathogenic strains lack P2c, and their growth is limited to individual locules. Likewise, production of proteinases (elastases) in culture has been correlated with isolate virulence to mice and with isolation from human hosts with aspergillosis (12, 13, 25), although soil isolates also produce proteases (17). Proteases may be required for virulence to plants, as resistance to *A. flavus* in corn kernels derives from a protease inhibitor (5).

Populations of *A. flavus* are highly polymorphic and complex (40), but we do not know if specialized subspecies or physiological races contribute to the broad host range (1, 6, 9). If specialization occurs, it seems not to include pathogen-host specificity (6). For example, Sussman (36) showed that diverse lepidopterans were infected by the same strain of *A. flavus* while isolates from one crop typically can infect other crops (3, 27). Lillehoj (15) proposed that host insects provide fungal dispersal and kernel damage sites for fungal colonization. The

ability of plant-derived isolates to infect insects, and vice versa, has not been determined.

Our objectives in this study were (i) to determine if *A. flavus* has pathogenic attributes lacking in *A. fumigatus* and *Aspergillus nidulans*, (ii) to determine if the ability to infect a host is correlated with the type of host from which a strain was isolated, and (iii) to determine if P2c pectinase and protease isozymes were correlated with pathogenic specialization. We found that, unlike *A. flavus*, strains of *A. fumigatus* and *A. nidulans* neither colonize living plant tissue nor kill living insects and that conidia of *A. flavus*, but not *A. fumigatus*, were resistant to digestion by insect hemocytes. However, strains of *A. flavus* were not host specific, nor was pathogenic specialization attributable to any of the enzymes assayed.

MATERIALS AND METHODS

Abbreviations. The abbreviations used in this paper are as follows: AFC, 7-amino-4-trifluoromethyl coumarin; DSI, disease severity index; EOMs, enzyme overlay membranes; IEF, isoelectric focusing; and Suc, succinyl.

Fungi. The isolates used in this study as well as their hosts and geographic origins are listed in Table 1.

Pectinase production on solid media. We measured pectinase production by the size and color of a clearance zone on a pectic screening medium (7). Spores of each isolate were inoculated onto the agar surfaces and incubated at 27°C. After 3 days, clearance zones were identified by staining with 0.05% ruthenium red for 1 h and then destaining for 30 min with distilled water. Clearance zones were produced by P2c, and red zones were produced by pectinesterase in the absence of P2c (7). Results are presented as + (clearing zones) and – (red zones).

Preparation of horse lung polymers. Fresh horse lungs were obtained from the Department of Veterinary Science at Cornell University, Ithaca, N.Y. The lungs were suspended in 0.85% NaCl–0.2% sodium tetraborate and homogenized in a Waring blender (full speed, 5 min), and insoluble polymeric material was collected by centrifugation (5,000 × g, 10 min). The lung material was washed until free of blood by 15 to 25 cycles of blending and centrifugation (as described above), leaving a light tan residue of insoluble lung polymers that was freeze-dried.

Preparation and analysis of culture filtrate. Standardized mycelial inocula (0.5 g [wet weight]) from 36-h Sabouraud dextrose cultures were incubated with shaking (100 rpm) at 30°C for 12 h in 15 ml of basal medium (containing the following liter⁻¹: 1 g of KH₂PO₄, 0.5 g of MgSO₄ · 7H₂O, 0.7 mg of Na₂B₄O₇ · 10H₂O, 0.5 mg of (NH₄)₆Mo₇O₂₄ · 4H₂O, 10 mg of Fe₂(SO₄)₃ · 6H₂O, and 0.3 mg of ZnSO₄ · 7H₂O [pH 6.5]) supplemented with horse lung polymers at 1% (wt/vol). Cultures were filtered through Whatman no. 1 filter paper (Maidstone, United Kingdom) and then through a 0.2-μm-pore-size Millipore filter unit (Molsheim, France) before being used for protease assays.

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TABLE 1. Protease activities, pectinase (P2c) activities, average DSIs, and time of death of *G. melonella* larvae infected with isolates of *Aspergillus*

<i>Aspergillus</i> species and strain	Host and geographic origin ⁱ	Protease ^a vs SUC-(Ala) ₂ -Pro-Phe-p-4-nitroamlide (mean $\pm$ SD)	Pectinase (P2c) ^b	DSI (and range) ^c	<i>G. melonella</i> mean time to death (h) ^d
<i>A. flavus</i>					
96-02 ^e	Human, USA	24 $\pm$ 3	+	2.3 (2-3)	26
96-03	Human, USA	38 $\pm$ 4	—	4.8 (4-5)	32
96-10	Human, USA	23 $\pm$ 4	—	4.8 (4-5)	26
96-17	Human, USA	96 $\pm$ 5	+	1.7 (1-3)	30
96-31	Human, USA	29 $\pm$ 4	+	2.3 (2-3)	24
96-36	Human, USA	51 $\pm$ 5	+	2.7 (2-4)	24
96-48	Human, USA	82 $\pm$ 6	+	1.7 (1-3)	32
96-58	Human, USA	30 $\pm$ 2	+	4.4 (4-5)	26
ARSEF 1003 ^f	Silkworm, Japan	28 $\pm$ 2	—	4.4 (4-5)	28
ARSEF 2157	Ant, New Guinea	85 $\pm$ 7	+	1.7 (1-2)	28
ARSEF 2818	Moth, Philippines	65 $\pm$ 4	+	4.0 (4-0)	34
ARSEF 2833	Moth, USA	48 $\pm$ 5	+	4.4 (4-5)	24
ARSEF 3144	Fly, Canada	39 $\pm$ 5	+	2.1 (2-3)	30
ARSEF 3430	Fly, Costa Rica	33 $\pm$ 6	+	4.2 (4-5)	31
SRRC 284	Cotton seed, USA	19 $\pm$ 4	+	3.8 (3-5)	42
SRRC 285	Cotton seed, USA	15 $\pm$ 1	+	3.3 (2-4)	38
SRRC 1000	Cotton seed, USA	2 $\pm$ 0	+	4.2 (3-5)	38
SRRC 1009	Cotton seed, USA	32 $\pm$ 3	+	4.2 (3-5)	32
SRRC 2045	Cotton seed, USA	84 $\pm$ 6	+	5.0 (5)	20
SRRC 2115	Cotton seed, USA	34 $\pm$ 3	+	3.5 (2-4)	24
TX 108 ^g	Corn, USA	26 $\pm$ 4	+	2.7 (2-3)	28
TX 118-3	Corn, USA	38 $\pm$ 5	+	3.2 (2-3)	32
TX 118-10	Corn, USA	26 $\pm$ 4	+	4.4 (4-5)	32
TX 119	Corn, USA	29 $\pm$ 4	+	5.0 (5)	42
TX 129	Corn, USA	27 $\pm$ 5	+	2.3 (2-4)	38
TX 131	Corn, USA	18 $\pm$ 3	+	4.0 (4)	28
c-span 16-1	Peanut, USA	7 $\pm$ 1	+	2.3 (2-3)	26
c-span 17-1	Peanut, USA	3 $\pm$ 1	+	2.7 (2-4)	28
c-span 25-1	Peanut, USA	2 $\pm$ 0	+	3.2 (2-4)	30
90-13-85	Peanut, USA	36 $\pm$ 5	—	4.0 (4-0)	34
<i>A. fumigatus</i> ^h					
4	Rat, USA	7 $\pm$ 1	+	1 (1)	
16424	Human, USA	7 $\pm$ 2	+	1 (1)	
28213	Cockatoo, England	3 $\pm$ 0	+	1 (1)	
42202	Human, France	3 $\pm$ 1	+	1 (1)	
<i>A. nidulans</i> A4 ⁱ	Saprophyte, USA	2 $\pm$ 0	—	1	

^a Mycelial inocula from Sabouraud dextrose cultures were incubated for 12 h in MM supplemented with horse lung polymers at (1%, wt/vol). Filtrates were assayed at 23°C in 10 mM Tris-HCl (pH 8)–4% (vol/vol) dimethyl sulfoxide with 0.06 mM substrate.

^b Plates containing pectic screening media were inoculated and incubated for 3 days. Enzyme activities are presented as + (P2c) and – (pectin esterase).

^c Five days after injection with fungal inoculum, bean leaf cuttings were rated on a DSI of 1 (no symptoms) to 5 (leaf death) (see text for complete scale).

^d Mean time to death of 20 larvae injected intrahemocoelically with 3,000 spores. *A. fumigatus* and *A. flavus* produced no mortalities.

^e Human *A. Flavus* strains (provided by Edward Bernard) were isolated from cases of aspergillosis at the Sloan Memorial Cancer Center in New York.

^f Insect *A. Flavus* strains were obtained from the USDA entomopathogenic fungus collection in Ithaca, N.Y.

^g Plant *A. Flavus* strains were provided by Nancy Keller, Texas A&M University.

^h *A. fumigatus* 4 (provided by Edward Bernard) was isolated from a laboratory at the Sloan Memorial Cancer Center in New York. *A. fumigatus* 16424, 28213, and 42202 were obtained from the American Type Culture Collection, Rockville, Md.

ⁱ *A. nidulans* A4 was obtained from the Fungal Genetic Stock Center at the University of Kansas Medical Center, Kansas City, Kans.

^j USA, United States of America.

Enzyme assays. Protease activity versus Suc-Ala-Ala-Pro-Phe-nitroanilide (Sigma, St. Louis, Mo.) was continuously measured spectrophotometrically in 10 mM Tris-HCl (pH 8) at 23°C (32). Assays were performed in duplicate. All results are representative of at least two similar experiments with different culture preparations.

IEF. Analytical IEF on ultrathin polyacrylamide gels was performed in an IEF cell (Bio-Rad, Hercules, Calif.) by using 1% ampholytes (Bio-Lyte 3/10; Bio-Rad) as previously described (33). For protease isoforms, gels were overlaid with gelatin overlays or EOMs impregnated with the fluorogenic substrate SUC-(Ala)₂-Pro-Phe-AFC (Enzyme Systems Products, Dublin, Calif.) by using procedures described previously (33). Gels were incubated at 37°C, and the appear-

ance of fluorescent bands was monitored by a long-wave (365 nm) UV lamp. The contact side of the membrane was visualized and photographed on thermal paper by using an EagleEye II image capture system (Stratagene, La Jolla, Calif.). The photographs were scanned with a flatbed scanner and Adobe Photoshop (Adobe Systems, Seattle, Wash.).

Leaf injection with fungal inoculum. Leaf cuttings of pinto bean cultivar G4523 were surface sterilized (0.25% sodium hypochlorite, 5 min, four changes of distilled water), put onto sterile agar medium (1.5% Noble agar), and injected in the middle vein with 2 μ l of the fungal inoculum (5×10^7 spores/ml) with a 30-gauge needle. Controls were injected with autoclaved fungal inoculum. Alternatively, inoculum (2 μ l containing 5×10^7 spores/ml) was applied to leaves

that had been injured either by touching the surface with a hot glass rod, which caused the tissue to become necrotic, or by placing a small piece of dry ice onto the surface, which caused the tissue to become chlorotic and flaccid. All cuttings were incubated at 22°C and 16 h light and 8 h dark, with a light intensity of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Five days after inoculation, the cuttings were rated on the following scale of 1 to 5: 1, green leaf without symptoms; 2, less than half of leaf showing yellowish discoloration; 3, approximately half of leaf showing yellowish or brown discoloration; 4, more than half of leaf showing discoloration; and 5, whole leaf discolored and necrotic.

The DSI of each isolate was expressed as the mean of ten leaves.

Systemic infections were identified by culturing pieces of infected leaves that had been dipped in 95% ethanol for a few seconds, surface disinfected as above, cut into 5-mm lengths, and incubated on Czapek Dox agar at 22°C for up to 5 days.

Infection of corn kernels. Ears of susceptible inbred strain Huffman (obtained from USDA Agricultural Research Service, Beltsville, Md.) were challenged with TX 118-3 and TX 129 (corn strains) and the eight human- and six insect-derived strains. The methods of inoculation were as follows: (i) inoculum (0.2 ml containing 2×10^6 conidia) applied to the surface of six uninjured seeds of each ear following removal of the husk and (ii) inoculum (10 μl containing 5×10^4 conidia) injected into a single seed in the midregion of each ear with a 30-gauge needle. Ears were placed individually in 3.1-liter rectangular boxes covered with plastic wrap. They were incubated at 22°C and 16 h light and 8 h dark, with a light intensity of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and examined for up to 2 weeks for fungal growth and rot in the inoculated and undamaged seeds. Results represent a minimum of at least three replicates.

Nonsymptomatic systemic infections of kernels were detected by removing uninjured kernels from inoculated areas and washing them briefly in acetone, and then 95% ethanol, to remove surface-associated propagules. Individual kernels were squashed, and samples of inner tissues were smeared on potato dextrose agar (Difco, Detroit, Mich.) to detect internal *A. flavus* spores and mycelium.

Pathogenicity of injected *A. flavus*, *A. fumigatus*, and *A. nidulans* toward the greater wax moth *Galleria mellonella*. Larvae of *G. mellonella* were purchased from the Sunfish bait company (Racine, Wis.) and fed on a *G. mellonella* artificial diet (Carolina Biological Supplies, Burlington, N.C.). Cohorts of fifth-instar larvae (mean weight, 200 ± 17 mg) were immobilized at 4°C for 30 min before injection. Conidia (3,000) in 10 μl of saline (0.85% NaCl) were injected intrahemocoelically into each caterpillar with a 30-gauge needle. Control larvae were injected with saline or with boiled (5 min) spore preparations. Larvae were kept on the diet at 22°C until moribund, when they were placed on water agar for 3 days to detect potential production of hyphae and conidia. Twenty larvae were injected per strain. Additional larvae were infected with 5,000 spores to document hyphal bodies in the hemocoel. Twenty microliters of hemolymph was harvested from each of 10 larvae and examined by using a Leica DMRB microscope (Leica Microscopes and Systems, Wetzlar, Germany).

The ability of strains of *A. fumigatus* and *A. nidulans* to colonize dead tissue was determined by freezing larvae and then injecting them with live spores as described above.

Materials. Reagents were procured as follows: SUC-(Ala)₂-Pro-Phe CH₂Cl and EOM components were from Enzyme Systems Products, and other enzyme substrates and inhibitors were from Sigma.

Statistical analysis. Strain differences in mean time to kill *G. mellonella* and DSI versus bean leaves were analyzed with the Statistical Analysis Software System (SAS Institute, Cary, N.C.). Treatment replicates for each isolate were first subjected to analysis of variance, followed by mean comparisons by the least-significant-difference test.

RESULTS

Inoculation of leaves. No symptoms (flaccidity, yellowing, or chlorosis) were observed on uninjured healthy leaves inoculated with any of the aspergilli strains. After 5 days, *A. flavus* could still be recovered by scraping a loop over the inoculation site and incubating the scrapings on Sabouraud dextrose agar. The leaves remained alive, and after a week most produced roots.

The mean DSI for bean leaves was not significantly different for human- (3.01), insect- (3.46), and plant-derived (3.61) isolates. Leaves injected with autoclaved spores of *A. flavus* remained symptomless.

The earliest symptoms occurred within 2 days of injection; bean leaves developed interveinal chlorosis followed by yellowing of tissue and a brownish necrosis. By 5 days postinoculation, the most aggressive strains had colonized the entire leaf and had sporulated. Leaves wounded by heat or dry ice and inoculated with *A. flavus* strains also underwent yellowing and

necrosis. Symptoms first developed at the site of the wound and then spread to healthy tissue. In some cases, 1 or 2 of the 10 leaves had delayed symptoms limited to yellowing at inoculation sites and chlorosis at the apex and edges of the leaves. These leaves were systemically colonized by *A. flavus*, and the fungus grew out from leaf pieces taken at locations 2 to 20 mm distance from the inoculation site. However the extent to which fungus grew into healthy tissue varied widely between replicates and between isolates.

The *A. nidulans* isolate produced small (less than 1.5 mm) wet lesions around puncture wounds, but no sporulation was observed. The four isolates of *A. fumigatus* produced little (small lesions) or no symptoms when injected into leaves.

Inoculation of corn. Little fungal colonization occurred on the surface of kernels when fungal inoculum was placed on undamaged kernels, and the appearance of the kernels did not change. *A. flavus* could not be recovered from asymptomatic surface-sterilized kernels.

All strains grew when they were injected directly into the kernel, although the size of the colonies on the ears varied in a qualitative manner. Growth of all strains of *A. fumigatus* and *A. nidulans* and *A. flavus* 96-02, 96-03, 96-31, 96-48, 2828, 3144, and 3430 was limited to the wounded kernel. *A. flavus* TX 118-3, TX 129, 96-17, 96-36, 96-58, 1003, 2818, and 2833 were more virulent; infected kernels were covered with conidia, and the underlying kernel was brown and rotten. These strains could spread to neighboring seeds, and TX 118-3, 96-17, and 96-58 caused widespread rot in some replicates.

Inoculation of insects. Conidia of *A. flavus* were not virulent when applied to the surface of healthy caterpillars. However, conidia from all strains were virulent (100% mortality within 48 h) when injected (3,000 spores per larvae) (Table 1). The average times to death following infection with plant (32 h; range, 20 to 42 h)-, insect (30 h; range, 24 to 34 h)-, and human (28 h; range, 24 to 32 h)-derived strains were not significantly different.

Within 1 day following death, white fungal hyphae began emerging through the cuticle, particularly at the intersegmental regions where the cuticle is thinnest. Within 72 h of host death, cadavers were covered in a thick coat of conidia. The progress of infection appeared to be similar for all 30 strains tested and, except for strain differences in spore color, so did the appearance of the sporulating cadavers.

Larvae injected with 0.85% saline or with boiled spores did not die. Boiled spores caused some darkening of a minority of larvae, indicative of a melanization defense response; however, larvae continued to feed normally. Injecting larvae with living spores of *A. fumigatus* or *A. nidulans* resulted in no deaths 5 days postinoculation. These fungi could, however, colonize insects killed by freezing and produced spores on cadavers within 4 days of injection.

The cellular defense system of insects infected with *A. flavus* 96-03, 96-17, ARSEF 3144, C-Span 17-1, and TX 118-3 and *A. fumigatus* 16424 were monitored by microscopic investigation of hemolymph. By 5 h postchallenge with each strain, many hemocytes contained large numbers of ingested spores, indicative of successful phagocytosis. By 16 h postchallenge, approximately 20% of phagocytosed spores were swollen and some of these had germinated with the hyphae emerging from disrupted hemocytes. At 24 h postchallenge, hyphal bodies were observed in the hemolymph, at which point the larvae were moribund. Following death, the body cavity of the cadaver rapidly filled with hyphal bodies. Spores of *A. fumigatus* were also rapidly phagocytosed but failed to germinate within hemocytes, and no hyphal bodies were observed free in the hemolymph.

FIG. 1. Analytical polyacrylamide IEF (pH 3 to 10) of proteinases produced by plant isolates (lanes: 1, strain 1000; 2, 16-1; 3, 2115; 4, 1009; and 5, 19-92), human isolates (lanes: 6, strain 96-31; 7, 96-48; and 8, 96-17), and insect isolates (lanes: 9, strain 2157; and 10, 1003) of *A. flavus* grown for 12 h in 1% horse lung polymer medium. Zymograms were prepared with gelatin overlays applied for 25 min (top row). The arrow indicates the major band that appeared after 5 min of incubation. EOM (bottom row) contained SUC-Ala-Ala-Pro-Phe-AFC for isoforms of subtilisin-like proteases. The membrane-gel sandwich was incubated at 37°C for 10 min.

Identification of *A. flavus* pectinases and proteinases. Of the 30 strains tested, only 4 (96-03, 96-10, ARSEF 1003, and 90-13-85) exhibited the red zone reaction on pectin-containing agar plates, indicative of the absence of P2c.

Production of subtilisin proteases was determined following a 12-h incubation in medium containing horse lung polymers (Table 1). Standardized mycelial inocula of each isolate were used to reduce the affects of differences in growth rate. The mean protease production by human-derived isolates (47 U; range, 23 to 96) was similar to that by the insect isolates (50 U; range, 28 to 85) but significantly greater ($P < 0.01$) than production by the plant isolates (25 U; range, 2 to 84). This difference reflected the very low enzyme levels produced by some, but not all, of the plant-derived isolates. The complexity of the proteases secreted by the 30 isolates of *A. flavus* during their growth on horse lung polymers was assessed on IEF gels with gelatin overlays (Fig. 1). Except for ARSEF 2157, all of the strains produced a major gelatin-degrading protease with a pI of ca. 8.5 which also hydrolyzed the subtilisin substrate SUC-(Ala)₂-Pro-Phe-AFC (Fig. 1). Incubating the IEF gel for extended periods of time revealed that all the isolates produced at least eight additional minor activities.

DISCUSSION

Conidia of plant-, insect-, and human-derived strains of *A. flavus* rapidly colonize leaves, kernels, and insects injured during inoculation but do not affect uninjured plant or insect material. Likewise, in nature, *A. flavus* can directly infect maize kernels under drought stress and high temperatures (32 to 36°C) known to compromise physiological defense systems as well as lead to cracks in the seed (16, 21, 30). In preliminary experiments we found that the condition of insect and plant material deteriorated considerably when maintained above 30°C. Consequently, we performed our studies at a compara-

tively low temperature (22°C) to maintain the condition of noncompromised hosts. Mechanical wounding by injection or necrosis should reproduce the compromising effects of dehydration and senescence on plant materials, as well as the effects of insect damage, a major facilitator of fungal attack (28, 31). Irrespective of their original host, most isolates of *A. flavus* produced similar symptoms when inoculated into wounded leaf tissue, suggesting that host association has not lead to divergence and differential adaptations.

About half of the strains isolated from humans and insects could spread between corn kernels. Several *A. flavus* strains with low virulence to cotton bolls lack P2c, and gene disruption has confirmed this enzyme's role in invasiveness of this host (29). The majority of strains isolated from human and insect hosts produced P2c, while the isolates lacking it were very destructive to bean leaf tissue, indicating that P2c's absence is not predictive of low invasiveness to all plant tissues. Thus, P2c may facilitate opportunistic exploitation of pectin but is not a specific adaptation for plant pathogenicity.

Wounds in insect cuticle increase susceptibility to opportunistic fungi (37). Insects efficiently eliminate most microorganisms from hemocoel circulation, but specialized entomopathogens avoid phagocytosis by masking their nonself nature (10, 34). Although hemocytes identified and phagocytosed conidia of *A. flavus*, these conidia still succeeded in germinating and producing hyphae. The *A. fumigatus* conidia did not germinate following phagocytosis but could colonize dead hosts, demonstrating the effectiveness of active insect defenses. *A. flavus* proliferated slowly within the hemocoel of the living insect but rapidly colonized killed hosts as a saprophyte and produced a thick coat of conidia on the cadavers available to infect new hosts. As with phytopathogenicity, virulence against insects varied but was not correlated with prior associations ($P > 0.235$).

IEF of *A. flavus* pectinases revealed a simple banding pattern comprising P1 and P2c, with P2c lacking in some strains (7). IEF of *A. flavus* proteinases reveals a much more complex banding pattern, although all strains produce a major isozyme that degrades the subtilisin substrate SUC-Ala-Ala-Pro-Phe-AFC. High and low protease producers had similar banding patterns. Thus, virulence to animal hosts may involve differences in the regulatory controls of protease gene expression rather than the profile of proteases produced. Low protease production does not preclude exploitation of animal hosts, as plant-derived strains that produced 50-fold less protease than strain 96-17 could still colonize insects. Gene disruption was successful in demonstrating a role for P2c in plant pathogenicity (29). If one protease can substitute for another, then gene disruption experiments to confirm that a subtilisin is required for virulence to mice would be expected to and have failed (18).

A. nidulans can cause only small lesions when inoculated into wounded plant tissues (8), even though it produces a broad spectrum of protein and polysaccharide-degrading enzymes (35). *A. nidulans* and *A. fumigatus* cannot extensively colonize living *Galleria* species and bean leaves. We were surprised that *A. fumigatus* was less aggressive than *A. flavus*, since *A. fumigatus* is the more frequent causative agent of human aspergillosis. Perhaps this difference is due in large part to physical characteristics; *A. fumigatus* has much smaller spores (2 μ m), which allow it to penetrate the lungs more effectively, than *A. flavus* (8 μ m) (22). Differences in spore size and aggressiveness suggest that the mode of pathogenesis of these two aspergilli toward humans differs and that efforts to block infection by one species may be ineffective for the other.

In this study, we demonstrate that most *A. flavus* strains can

cause disease in both plants and animals. Many fungi moved from opportunistic forms to specialized pathogens by gaining the ability to produce host-selective toxins that provided the genetic isolation for evolutionary change (26, 38). Although *A. flavus* produces a variety of toxins, including aflatoxins (19, 39), the routine association of *A. flavus* with various plants and insects in an opportunistic fashion, as these nutritional resources temporarily become available, could explain why populations of *A. flavus* have not diverged into separate pathogenicity types. It is possible that *A. flavus* routinely infects both plants and animals with the insect acting as vector (2). In this scenario, the insect eventually serves as a substrate to create a very large inoculum to exploit insect damage in the plant.

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