Identification of Atoxigenic *Aspergillus flavus* Isolates to Reduce Aflatoxin Contamination of Maize in Kenya

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Abstract

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Aspergillus flavus has two morphotypes, the S strain and the L strain, that differ in aflatoxin-producing ability and other characteristics. Fungal communities on maize dominated by the S strain of *A. flavus* have repeatedly been associated with acute aflatoxin poisonings in Kenya, where management tools to reduce aflatoxin levels in maize are needed urgently. *A. flavus* isolates (n = 290) originating from maize produced in Kenya and belonging to the L strain morphotype were tested for aflatoxin-producing potential. A total of 96 atoxigenic isolates was identified from four provinces sampled. The 96 atoxigenic isolates were placed into 53 vegetative compatibility groups (VCGs) through complementation of nitrate non-utilizing mutants. Isolates from each of 11 VCGs were obtained from more than one maize sample, isolates from 10 of the VCGs were detected in multiple districts, and isolates of four VCGs were found in multiple provinces. Atoxigenic isolates were tested for potential to reduce aflatoxin concentrations in viable maize kernels that were co-inoculated with highly toxigenic S strain isolates. The 12 most effective isolates reduced aflatoxin levels by >80%. Reductions in aflatoxin levels caused by the most effective Kenyan isolates were comparable with those achieved with a United States isolate (NRRL-21882) used commercially for aflatoxin management. This study identified atoxigenic isolates of *A. flavus* with potential value for biological control within highly toxic *Aspergillus* communities associated with maize production in Kenya. These atoxigenic isolates have potential value in mitigating aflatoxin outbreaks in Kenya, and should be evaluated under field conditions.

Aflatoxins are a series of highly toxic polyketides produced by several species of *Aspergillus* (46,65). The most commonly occurring aflatoxin, aflatoxin B_1, is a genotoxin known to be carcinogenic and teratogenic for both humans and animals (49,69) and, to date, the only mycotoxin classified as a group 1a human carcinogen by the International Agency for Research on Cancer (40,41). Crops infected by aflatoxin-producing fungi frequently become contaminated with aflatoxins. Aflatoxin contamination results in reduced crop value and diminished health of humans and domestic animals that consume the contaminated crops (72). The quantity of ingested aflatoxins determines whether health effects are chronic (e.g., immune suppression, impaired child growth, abnormal fetal development, and cancer) or acute (e.g., hepatitis and jaundice, abdominal swellings, and death) (11,32,36,43,71). To date, Kenya is the only nation with a population that has repeatedly experienced epidemics of acute aflatoxicosis (5,53,55). These episodes resulted from consumption of highly contaminated, homegrown maize and have extended over two decades, with the most recent occurring from 2004 through 2006, when several hundred Kenyans died from acute aflatoxin poisoning in several districts of the Eastern Province (12,54). During these periods, many thousands of individuals were exposed to unsafe aflatoxin levels (54,60). In 2010, another extensive epidemic occurred in Kenya, with high frequencies of harvested maize lots containing levels of aflatoxins unfit for human consumption (42).

Aspergillus flavus is the most frequently implicated causal agent of aflatoxin contamination of maize (46). This species has several morphotypes (commonly called strains), among which the L and S strains are most studied. These strains differ in several characteristics, including production of sclerotia, conidia, and aflatoxins (14). L strain isolates produce few, large sclerotia (average >400 μm) and highly variable quantities of aflatoxins, with some isolates (called atoxigenic isolates) entirely lacking the ability to produce aflatoxins. In contrast, S strain isolates produce numerous, small sclerotia (average <400 μm) and, on average, higher levels of aflatoxins than L strain isolates (14). Each of the morphotypes is further subdivided into many vegetative compatibility groups (VCGs) delineated by a heterokaryon incompatibility system (57). There is also variability among VCGs in aflatoxin-producing ability. Thus, *A. flavus* exists in complex communities that vary widely in both strain and VCG composition and aflatoxin-producing ability. Fungal communities in Kenya associated with severe maize contamination and deaths in human populations have atypical structures, with the S strain of *A. flavus* highly dominant, and increasing incidence of the S strain associated with increasing contamination levels (59,60).

The influence of aflatoxins on human populations in Kenya over the past decade demonstrates a clear need for tools to manage contamination of locally produced maize. A highly promising method for aflatoxin management has been the use of atoxigenic isolates of *A. flavus* to competitively exclude aflatoxin producers and, thereby, reduce aflatoxin concentration in a crop (21,25,29). Two atoxigenic isolates used commercially in the United States are very effective at inhibiting aflatoxin contamination by the S strain of *A. flavus* (35). Identification of atoxigenic isolates of *A. flavus* native to Kenya might provide an environmentally sound, ecologically adapted, native, biological resource useful in mitigating aflatoxin contamination of maize produced in Kenya. This study sought to determine whether atoxigenic isolates of *A. flavus* with potential value in biological control could be selected from highly toxic fungal communities in aflatoxin-contaminated maize produced in Kenya that had been associated with lethal aflatoxicosis.

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Materials and Methods

Fungal isolation, maize samples, and fungal inoculum preparation. Isolates of *A. flavus* were collected during previous studies

(59,60). Briefly, samples of maize kernels (n = 263) were collected from farmers in 12 districts in four provinces (Eastern, Coast, Rift Valley, and Nairobi) during 2004, 2005, and 2006 (Fig. 1). Mean sample weight was 290 g (range of 111 to 430 g/sample). The maize was finely ground in a laboratory hammer mill (IKA Labortechnik, Heitersheim, Germany), and fungal isolates were recovered by dilution plate technique on modified rose Bengal agar (M-RBA), as described previously (17,60). After cultivation at 31°C for 5 days on 5/2 agar (5% V8 vegetable juice, 2% agar, pH 5.2), *Aspergillus* section *Flavi* isolates were identified to species (47,48) and strain based on macroscopic and microscopic characteristics, and stored in sterile water (four to six colonized 3-mm-diameter agar plugs added to 2.5 ml of sterile water) at 8°C for the working cultures and on silica gel at 8°C for long-term storage. To produce fungal inoculum, each L strain isolate was grown on 5/2 agar for 5 days at 31°C in the dark. Conidia were transferred to glass vials containing 20 ml of sterilized, deionized water using cotton swabs. Conidial concentrations were measured with a turbidity meter (Model 965-10; Orbeco-Hillige, Farmingdale, NY), calculated using the nephelometric turbidity unit (NTU) versus CFU curve: $Y = 49{,}937X$, where X = NTU and Y = conidia/ml. The conidial concentration for each isolate was adjusted to 10^6 conidia/ml.

Identification of atoxigenic isolates associated with maize produced in Kenya. Aflatoxin-producing ability of each of the *A. flavus* L strain isolates was determined using autoclaved maize kernels (10 g per 250-ml Erlenmeyer flask). Maize cv. 33F88 (Pioneer Hi-Bred International Inc., Johnston, IA) was used for all experiments. Each flask was sealed with a BugStopper (Whatman, Piscataway, NJ) and autoclaved at 121°C for 60 min. After autoclaving, maize moisture level was quantified with an HB43 Halogen Moisture Analyzer (Mettler Toledo, Columbus, OH) and adjusted to 25%. Each autoclaved maize sample was then inoculated with 1 ml of spore suspension (10^6 conidia/ml water) of the appropriate isolate and incubated for 7 days at 31°C in the dark. After incubation, the maize cultures of *A. flavus* were processed as described previously (19,60). Briefly, the colonized maize for each sample was blended in 80% methanol (50 ml) until evenly homogenized, and the blended mixture was filtered through Whatman no. 4 filter paper. Culture filtrates were spotted directly onto thin-layer chromatography (TLC) plates (Silica gel 60; EMD, Darmstadt, Germany) adjacent to aflatoxin standards (Aflatoxin Mix Kit-M; Supelco, Bellefonte, PA). The plates were developed in ethyl ether/methanol/water (96:3:1), air dried, and the aflatoxins were visualized under 365 nm UV light. Aflatoxins were quantified directly on TLC plates with a scanning densitometer (TLC Scanner 3; Camag Scientific Inc., Wilmington, NC). Filtrates that initially tested negative for aflatoxins were combined with 50 ml of water and extracted twice serially with methylene chloride (25 ml). Methylene chloride extracts were passed through a layer of anhydrous $NaSO_4$, evaporated to dryness, and dissolved in an appropriate volume of methylene chloride for accurate densitometry. Aflatoxins were then separated on TLC plates and quantified as described above. Isolates that produced levels of aflatoxins below the limit of detection (aflatoxin B_1 at 0.5 ng/g) were considered atoxigenic and evaluated further as potential biocontrol agents. Each L strain isolate of *A. flavus* was tested for atoxigenicity at least three times.

Aflatoxin production during co-infection of viable maize kernels with toxigenic and atoxigenic isolates of *A. flavus*. Each atoxigenic L strain isolate of *A. flavus* (n = 96) was initially evaluated on viable maize kernels for ability to interfere with aflatoxin production by a highly toxigenic *A. flavus* S strain isolate (FGSC A1169). Isolate FGSC A1169 was previously obtained from a highly contaminated maize sample associated with the 2004 outbreak of acute aflatoxicosis in Kenya (59). Prior to inoculation, undamaged maize kernels of Pioneer 33F88 were surface-sterilized by submerging the kernels in hot water for 45 s at 80°C (51), air dried for approximately 1 min on an aseptic surface in a biological safety cabinet, and distributed into sterilized glass flasks (10 g of maize per 250-ml flask). Flasks were plugged with BugStoppers to prevent humidity loss and allow gas exchange. Moisture content of the surface-sterilized maize kernels was determined as described above. Efficiency of surface sterilization and ability of kernels to germinate were monitored by plating three randomly selected kernels from each flask onto a selective agar medium (M-RBA) (17), followed by incubation at 31°C in the dark for 14 days. Greater than 90% of the kernels germinated and fungal contaminants were not observed at the end of the incubation period. Equal inocula (2×10^5 conidia/isolate/flask) of S strain (aflatoxin-producing) and atoxigenic L strain isolates were mixed and added to the flasks (0.75 to 0.80 ml/flask depending on kernel moisture content), which were then gently agitated to coat the kernels with the inoculum. Kernel moisture after inoculation was 25%. Each *A. flavus* L strain isolate was tested using four replicates. Inoculated maize was incubated at 31°C for 7 days in the dark. The experiment was then terminated by addition of 50 ml of 70% methanol, and aflatoxins were extracted following the protocol outlined above. Inhibition of aflatoxin contamination in these co-inoculation experiments was calculated as a percentage of the aflatoxin content in kernels inoculated with only the aflatoxin-producing isolate (FGSC A1169).

Initial evaluation of the 96 isolates was a screen to select isolates for further testing. Thus, the ability of each of the 96 atoxigenic L strain isolates to interfere with aflatoxin production by FGSC A1169 was tested in at least a single comparison of 15 to 20 isolates at a time. Some isolates were included in up to five comparisons. Each completely randomized experiment was replicated three times. Isolates (n = 13) that either performed well (>80% less aflatoxin B_1 contamination than the control samples) in the initial screen or that were associated with >50% less aflatoxin contamination and were members of VCGs isolated from two or more locations in Kenya (n = 10) were evaluated further. The thresholds of 80 and 50% less aflatoxin contamination, respectively, were selected on an arbitrary basis to reduce

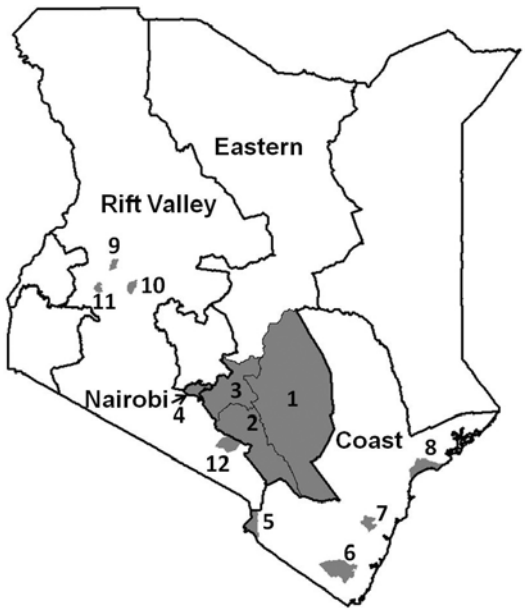

Fig. 1. Sites in four provinces and 12 districts of Kenya from which maize samples were collected and isolates of *Aspergillus flavus* obtained. Maize samples from Eastern and Nairobi Provinces were associated with acute aflatoxicoses outbreaks in 2004 to 2006. Maize samples from the Rift Valley and Coast Provinces were collected in 2006 to evaluate *Aspergillus* communities in maize-growing areas adjacent to the aflatoxicosis outbreak regions (59,60). Eastern Province: District 1 = Kitui, 2 = Makueni, and 3 = Machakos; Nairobi Province: District 4 = Nairobi; Coast Province: District 5 = Taita Taveta, 6 = Kwale, 7 = Kilifi, and 8 = Tana River; Rift Valley Province: District 9 = Marakwet, 10 = Baringo, 11 = Uasin Gishu, and 12 = Kajiado.

the number of isolates for further evaluation in the preliminary experiments (Table 1).

Five *A. flavus* L strain isolates (C6-E, C8-F, E62-L, E63-I, and R1-N) most effective at reducing aflatoxin contamination of maize kernels in the preliminary experiments (Table 1) were tested further for consistency in efficacy against two S strain isolates, FGSC A1169 and ATCC MYA-384. This was done using the assay described above in two additional experiments, one with each S strain isolate, and each experiment included four replicates per treatment (Table 2). In these experiments, comparisons were made with NRRL-21882, the atoxigenic isolate that is the active ingredient in AflaGuard (Syngenta, Wilmington, DE), a biocontrol product currently registered for management of aflatoxins in maize in the United States.

Table 1. Influence of co-inoculation with atoxigenic L strain isolates of *Aspergillus flavus* from four provinces in Kenya on the aflatoxin content of viable maize kernels infected by an aflatoxin-producing isolate of the S strain morphotype of *A. flavus*

		Aflatoxin B_1 (µg/g)[w]		
Prov., isolate[x]	VCG[y]	Exp 1	Exp 2	Avg. diff. (%)[z]
Eastern				
E971-E	…	199 a	253 a	…
Coast				
C6-E	KN00A	21 bc	41 de	86.6 ± 4.0
C2-J	KN00B	19 c	49 bcde	85.5 ± 7.0
C8-F	KN012	28 bc	37 de	85.7 ± 0.5
C5-K	KN006	28 bc	52 bcde	82.7 ± 4.5
C3-G	KN015	23 bc	66 bcd	81.2 ± 10.2
Eastern				
E63-I	KN001	17 c	31 e	89.6 ± 2.8
E62-L	KN00C	18 c	37 de	88.2 ± 4.0
E266-D	KN004	nd	48 de	81.0 ± 5.3
E836-A	KN00D	39 bc	54 bcde	79.5 ± 1.5
E138-A	KN015	49 abc	53 bcde	77.2 ± 2.8
E987-H	KN00E	38 bc	72 bcd	76.2 ± 6.8
E103-G	KN00F	54 abc	53 bcde	76.0 ± 4.4
E54-I	KN002	50 abc	62 bcde	75.2 ± 0.5
E916-O	KN008	57 abc	81 bcd	69.7 ± 2.3
E804-C	KN00G	nd	92 b	63.6 ± 18.0
Rift Valley				
R1-N	KN00H	21 bc	40 de	86.8 ± 3.8
R7-K	KN012	nd	39 de	84.6 ± 7.0
R8-F	KN00I	nd	44 cde	82.6 ± 11.5
R1-J	KN00J	31 bc	62 bcde	80.0 ± 6.3
R7-H	KN011	50 abc	55 bcde	76.6 ± 2.7
R5-R	KN00K	51 abc	55 bcde	76.3 ± 2.7
R8-P	KN007	nd	69 bcd	72.7 ± 25.5
R8-C	KN007	94 ab	64 bcde	63.7 ± 15.4

[w] Isolates with mean aflatoxin B_1 values followed by some of the same letters within a column do not differ significantly according to Tukey's honestly significant difference test ($P = 0.05$) (56); nd = no data available. Isolate was not included in experiment 1.

[x] Province and isolates. All isolates originated from maize samples collected in Kenya from 2004 to 2006. Isolates did not produce aflatoxins except for isolate E971-E (FGSC A1169) which produced aflatoxin B_1 at >100 µg/g of maize and belongs to the S strain morphotype of *A. flavus*. Results for E971-E indicate the aflatoxin value for kernels inoculated with that isolate alone. Other isolates were co-inoculated with E971-E to assess capacity to interfere with aflatoxin contamination during co-infection.

[y] VCG = vegetative compatibility group. VCGs with a letter (A to K) indicate isolates that belonged to a single-member VCG (VCGs for which only one isolate was detected). VCGs with a number (KN001 to KN0015) indicate isolates that belonged to multiple-member VCGs (VCGs for which more than one isolate was detected).

[z] Average difference (mean ± standard deviation) in aflatoxin levels (%) = [1 – (total aflatoxin content in maize co-inoculated with both toxigenic and atoxigenic isolates of *A. flavus*/total aflatoxin content in maize inoculated with only isolate E971-E)] × 100. Standard deviations for isolates included in both tests were calculated based on the difference in aflatoxin levels (%) obtained in the two experiments. Standard deviations for the five isolates that were only included in experiment 2 (E266-D, E804-C, R7-K, R8-F, and R8-P) were calculated based on differences in aflatoxin levels (%) for the four replicates of each treatment.

VCG analysis. All 96 atoxigenic L strain isolates of *A. flavus* were subjected to VCG analyses in order to assess diversity among the atoxigenic isolates and to determine which VCGs are most common in the Kenyan maize samples evaluated and, thus, potentially well adapted to maize cultivation in Kenya. In summary, mutations in any one of several genes in the nitrate-reductase pathway can produce nitrate nonutilizing (*nit*$^-$) mutants. Mutants affected in different genes can complement each other when paired on starch complementation medium at 31°C (26), and restore wild-type growth. In this study, *nit*$^-$ mutants were generated for each atoxigenic isolate on M-RBA medium containing chlorate, as described previously (18). Complementary tester mutants (*cnx*$^-$ and *niaD*$^-$) were developed (6,7), and the ability of a *nit*$^-$ mutant from each isolate to complement one or both tester mutants was evaluated. Isolates yielding *nit*$^-$ mutants that did not complement one of the tester mutants within 10 days were considered to belong to a VCG other than the one defined by the tester pair, while those complementing one of the tester mutants were considered to belong to the same VCG as the tester pair.

Statistical analyses. Randomized complete block designs with three to four replicates were used in all experiments. Aflatoxin concentration was log transformed and subjected to analysis of variance (ANOVA). ANOVA was performed with the general linear model procedure of SAS (version 9.2; SAS Institute, Cary, NC). Mean separations were performed on data from experiments with statistically significant ($P = 0.05$) differences, using Tukey's honestly significant difference test (56). Mean differences in aflatoxin levels (percent difference between inoculated maize and control maize treatments) were calculated as [1 – (total aflatoxin content in maize co-inoculated with both toxigenic and atoxigenic isolates of *A. flavus*/total aflatoxin content in maize inoculated with the S strain isolate alone)] × 100. Standard deviations of mean differences in aflatoxin levels were calculated as a measure of variability in efficacy. All analyses and calculations were performed with SAS.

Results

Identification of atoxigenic isolates of *A. flavus*. More than 3,400 *A. flavus* isolates were recovered from 263 ground maize samples collected in 2004 to 2006 from four provinces of Kenya. Detailed descriptions of the samples and general characteristics of the fungal isolates have been published (59,60). The *A. flavus* morphotypes were very unevenly distributed among provinces, with the L strain composing 27% of the *A. flavus* isolates from the Eastern Province compared with 88% of the isolates from the Rift Val-

Table 2. Aflatoxin B_1 levels produced following co-infection of viable maize kernels by atoxigenic L strain isolates of *Aspergillus flavus* that originated from Kenya or the United States (NRRL-218820) and two highly toxigenic S strain isolates from Kenya

	Aflatoxin B_1 (µg/g)[x]		
Isolate[y]	Exp 1	Exp 2	Avg. diff. (%)[z]
None	105.4 a	109.2 a	…
62-L	8.4 b	19.0 b	87.3 ± 6.7
C6-E	8.8 b	20.6 b	86.5 ± 7.4
NRRL-21882	9.3 b	23.1 b	85.1 ± 8.7
R1-N	11.5 b	25.0 b	83.1 ± 8.4
63-I	12.4 b	25.3 b	82.6 ± 8.1
C8-F	12.7 b	26.8 b	81.7 ± 8.8

[x] Mean values followed by a common letter within a column do not differ significantly according to Tukey's Honestly Significant Difference test ($P = 0.05$) (56).

[y] Isolate co-inoculated. For None, two S strain isolates of *A. flavus* were used. Experiment 1: isolate FGSC A1169; experiment 2: isolate ATCC MYA-384. The corresponding aflatoxin concentration was obtained from maize kernels inoculated with the S strain isolate alone.

[z] Average difference (mean ± standard deviation) in aflatoxin concentrations (%) = [1 – (total aflatoxin content in maize co-inoculated with both toxigenic and atoxigenic isolates of *A. flavus*/total aflatoxin content in maize inoculated with the S strain isolate alone)] × 100.

ley Province and 91% of the isolates from the Coast Province (60). Greater numbers of isolates were obtained from the Eastern Province because of the long history of lethal aflatoxicosis in that province (60) and the resulting urgent need for aflatoxin management options in that region. Of the 290 L strain isolates screened for aflatoxin-producing ability on autoclaved maize (222 from the Eastern Province, 32 from the Coast Province, and 36 from the Rift Valley Province), 96 were identified as atoxigenic based on a detection limit for total aflatoxins of 0.5 μg/kg of maize (*data not shown*). Aflatoxin B_1 production levels of the remaining 194 isolates ranged from 0.5 to >400,000 μg/kg of maize (ppb).

Co-infection of viable maize kernels with toxigenic and atoxigenic isolates of *A. flavus*. All 96 atoxigenic *A. flavus* L strain isolates were associated with lower maize aflatoxin content when co-inoculated with a highly toxigenic *A. flavus* S strain isolate (FGSC A1169) compared with maize kernels inoculated with the S strain isolate alone (*data not shown*). Average reductions in aflatoxin B_1 concentration ranging from 7.1 ± 4.4 to 98.3 ± 8.0% (mean of 59.2 ± 22.9% for all 96 isolates) were observed in co-inoculated maize kernels compared with maize kernels inoculated with FGSC A1169 alone. In all, 23 isolates from the initial screens were chosen for further tests. Aflatoxin B_1 levels in co-inoculation experiments conducted with the 23 selected isolates were significantly lower in both experiments. Average aflatoxin B_1 levels were 63.6 to 89.6% less in co-inoculated treatments compared with the control treatments with an S strain isolate alone (mean of 79.3% reduction for all 23 isolates; Table 1).

Co-inoculation of maize kernels with each of the five Kenyan atoxigenic L strain isolates selected for further evaluation with the atoxigenic isolate NRRL-21882 demonstrated significantly lower aflatoxin levels than in kernels inoculated with an S strain isolate alone (Table 2). The five isolates from Kenya performed similarly during the additional evaluations as in the preliminary experiments, causing an average of 87.4 ± 2.4% less aflatoxin in the preliminary tests (Table 1) and 84.4 ± 1.5% less aflatoxin in the additional evaluations (Table 2). All atoxigenic isolates were statistically similar in ability to interfere with aflatoxin production in viable maize kernels regardless of which S strain isolate was used in the experiment (Table 2).

VCG analysis. All 96 atoxigenic *A. flavus* isolates obtained from maize produced in Kenya were successfully placed into a VCG by complementation of *nit*⁻ auxotrophs. A total of 53 VCGs was detected. However, 41 of the VCGs were each represented by a single atoxigenic isolate. Of the atoxigenic isolates, 50% belonged to one of 12 multimember VCGs. Seven of the multimember VCGs were detected in the Eastern Province, seven in the Coast Province, and four in the Rift Valley Province. Isolates in four of the VCGs were detected in two provinces and one isolate (in VCG KN008) was detected in six districts, three provinces, and seven maize samples (Table 3). These included four districts that are targets for aflatoxin management because of recent health or economic impacts from aflatoxin contamination of maize: Kitui, Machakos, Makueni, and Tana River.

Discussion

Aflatoxins impact both the safety and value of foods and feeds and, as such, management of aflatoxin contamination of food and feed is required both to capture optimal markets and to maintain food security (72). In developing nations, economic incentives for management of aflatoxin contamination are typically reduced due to less stringent regulatory oversight (72). However, in recent years, public awareness campaigns, news reports of human health effects, and episodes of crop destruction have greatly increased incentives for aflatoxin management even in very-low-income regions (34,42). Nowhere have aflatoxins been more in the public eye than in Kenya, where severe aflatoxin contamination of maize has caused human deaths repeatedly over the past three decades (12,55,59). In Kenya, crop destruction is a devastating aflatoxin management option. Crops may be a farmers' only possession of cash value, and crop destruction can result in impoverishment and malnourishment. Farmers in food-scarce regions are in urgent need of affordable management strategies to protect consumers from the harmful effects of aflatoxins and to optimize crop value.

Development of technologies to prevent or reduce aflatoxin contamination of maize has been a complex and frustrating goal for over three decades. Proper drying and storage of grains like maize can prevent postharvest increases in aflatoxin content (66,73) but driers and storage facilities are not available to all growers, and timely harvest and drying of grain can sometimes be prevented by adverse weather conditions that promote contamination of harvested grains. Furthermore, significant quantities of aflatoxins can develop in grain prior to harvest. Cultivars resistant to aflatoxin contamination by *A. flavus* have been sought for at least 30 years (9,39,50,68,70). However, commercially available maize cultivars

Table 3. Occurrence of single and multiple member vegetative compatibility groups (VCGs) among *Aspergillus flavus* isolates obtained from maize samples collected from four maize growing provinces in Kenya in 2004 to 2006

	VCG[w]												
Province, district[x]	**KN001**	**KN002**	**KN004**	**KN005**	**KN006**	**KN007**	**KN008**	**KN010**	**KN011**	**KN012**	**KN013**	**KN015**	**Single**
Eastern													
Kitui	+	+	+	+	…	…	+	…	…	…	…	+	+
Machakos	…	…	…	+	…	…	+	…	…	…	…	…	+
Makueni	+	+	+	…	…	…	+	…	…	…	…	…	+
Nairobi													
Nairobi	…	…	+	…	…	…	…	…	…	…	…	…	+
Coast													
Taita Taveta	…	…	…	…	+	…	…	+	+	…	+	…	+
Kwale	…	…	…	…	…	…	…	…	…	…	…	+	+
Tana River	…	…	…	…	+	…	+	…	+	…	…	…	+
Kilifi	…	…	…	…	…	…	+	…	…	+	…	…	+
Rift Valley													
Marakwet	…	…	…	…	…	+	…	…	…	+	…	…	+
Baringo	…	…	…	…	…	+	…	…	…	…	…	…	+
Kajiado	…	…	…	…	…	+	+	…	…	…	…	…	+
Uasin Gishu	…	…	…	…	…	+	…	…	+	+	…	…	+
No. of samples[y]	2	2	4	2	3	4	7	1	3	5	2	3	33
No. of atoxigenic isolates[z]	3	5	4	2	3	11	7	2	4	10	2	3	41

[w] Presence (+) of isolates of specific multiple-member VCGs (KN001 to KN015, VCGs for which more than one isolate was detected) and single-member VCGs (41 VCGs for which only a single isolate was detected) in each district.
[x] District within the province.
[y] Number of maize samples from which the indicated VCG of *A. flavus* was isolated. Total number of samples was 156.
[z] Number of atoxigenic *A. flavus* isolates detected in the indicated VCG.

labeled as aflatoxin resistant still are not available in the United States, where such cultivars have been sought most intensively (27,38,39,70). A biological control strategy that utilizes naturally occurring, atoxigenic isolates of *A. flavus* to competitively exclude aflatoxin producers was originally a controversial proposal (16,21,45) thought to lack practical value. However, this strategy has received farmer acceptance and support in the United States (63,64), and currently provides the only preharvest management tool with documented efficacy in commercial agriculture (1,4,13,30,31,74). In North America, this biological control strategy is currently the basis for the only aflatoxin management products registered for commercial preharvest use on any crop (29,44). Although commercial use of atoxigenic *A. flavus* began in the United States (16), developing nations with reduced infrastructure and high consumption of maize and groundnut may be where biocontrol of aflatoxins has the greatest potential. Indeed, atoxigenic isolate formulations have long-term stability under nonrefrigerated conditions, a trait useful in regions where refrigeration is unavailable or expensive (8).

Aspergillus communities associated with acute aflatoxicosis and human death in the Eastern Province of Kenya have some of the highest aflatoxin-producing potentials ever reported (59,60). This study demonstrated that, even within these highly toxic fungal communities, there reside atoxigenic isolates of potential value in the management of aflatoxins. Communities composed of highly toxigenic fungi have proportionally fewer atoxigenic isolates (60) and, thus, screening for atoxigenic isolates from such communities may be time and cost intensive. Nonetheless, 48 of the 96 identified atoxigenic L strain isolates of *A. flavus* were isolated from highly contaminated maize samples (total aflatoxins at up to 4,400 µg/kg of maize) originating from acute aflatoxicosis outbreak regions in Kenya in 2004 to 2006. These isolates represent a small fraction of the L strain population of *A. flavus* which, likewise, are a small fraction of the total *Aspergillus* community in the Eastern Province of Kenya (60). Experience in the United States has shown that appropriately timed application of similar rare, atoxigenic isolates of *A. flavus* results in fungal communities dominated by the applied atoxigenic isolates, resulting in greatly reduced aflatoxin-producing potential of the affected fungal communities (20,21). Since 2007, hundreds of isolates of *A. flavus* have been screened, first by morphological criteria to remove highly toxigenic S strain isolates, then by quantifying aflatoxin production during in vitro culture on sterilized maize kernels and, finally, by testing the ability of the atoxigenic isolates to interfere with aflatoxin contamination of viable maize kernels by toxigenic isolates in laboratory assays. The selected atoxigenic isolates are now ready for field testing. Successful collection of atoxigenic isolates of potential value in aflatoxin management from maize samples associated with lethal aflatoxicosis in the Eastern Province suggests that valuable atoxigenic isolates may similarly be found in *Aspergillus* communities resident in other areas where improved aflatoxin management is of concern.

A. flavus is composed of many VCGs. VCGs evolve as clonal lineages (33,37) and aflatoxin production is more similar within VCGs than among VCGs. Some VCGs of *A. flavus* are composed of only atoxigenic members (7). The VCGs reported in this study provide potentially valuable fungal germplasm for development of biological control agents directed at limiting contamination of maize in Kenya. Atoxigenic isolates are frequently monitored in the environment by VCG analyses (28,58). In order to perform VCG analyses in this study, auxotroph tester pairs were developed and are now available for atoxigenic isolate monitoring during field testing in Kenya. Although the atoxigenic isolates represent 53 locally adapted genetic groups of *A. flavus*, they may represent a small subset of the overall genetic diversity of atoxigenic isolates of *A. flavus* endemic to Kenya. Diverse fungal germplasm may be of value in overcoming problems with biocontrol agents (e.g., loss of efficacy) or for assembling complex mixtures of atoxigenic isolates for increased efficacy across complex cropping systems and for long-term displacement of highly toxigenic *Aspergillus* isolates. A comprehensive collection of *A. flavus* isolates from across Kenya would increase genetic resources for development of such strains.

Atoxigenic isolates of *A. flavus* can prevent unacceptable aflatoxin levels in crops through both direct interference with aflatoxin production during co-infection and modification of *A. flavus* population composition by competitive exclusion. The first process involves both competitive exclusion during host tissue invasion and disruption of aflatoxin biosynthesis through an as-yet-undefined process (22,51). In this study, atoxigenic isolates were compared for interference with aflatoxin contamination of maize kernels co-infected by a highly toxigenic S strain isolate of *A. flavus*. This type of evaluation has been used (1,4,10,24,51) to compare efficacy of atoxigenic isolates since such isolates were first evaluated as potential biocontrol agents (15). However, this type of screen may not provide the most useful insights for selecting optimal atoxigenic isolates of *A. flavus* for biocontrol because competitive exclusion of aflatoxin producers during crop production probably contributes more to both single-season and long-term efficacy (21). Competitive exclusion is dependent on isolate reproduction and competition in the local environment (26). Isolates of *A. flavus* that produce the most conidia dominate *A. flavus* communities and have proportional influences on contamination regardless of ability to compete during host tissue invasion (51). Competitive exclusion of aflatoxin producers during epidemic increases allows atoxigenic isolates to compensate for uneven application and to have influences beyond treated areas, potentially influencing compositions of fungal communities in nearby, untreated fields (13). This is a biocontrol benefit not shared with chemical pesticide applications.

Atoxigenic isolates of *A. flavus* in VCGs identified in this study all belong to the L strain morphotype of *A. flavus* (14), similar to L strain VCGs from other regions (14,58). However, *A. flavus* VCGs can evolve independently for long periods (33,37). Even within morphotypes, there is great variability among VCGs in adaptive traits (7,25,51), including aflatoxin producing ability, virulence, competitiveness, sensitivity to antibiotics, tolerance to soil conditions, and life strategy. Phenotypic variability suggests differential adaptation among isolates of different VCGs to various ecological niches (23,25). The diversity of niches to which isolates of specific VCGs identified in this study are adapted is not yet known. Introduction and application of exotic microorganisms can bear considerable risk to animals, plants, and the environment (62,67). It has been suggested that atoxigenic *A. flavus* isolates originating from North America be introduced to various African countries for aflatoxin management because the North American isolates are components of biopesticides currently registered with the United States Environmental Protection Agency (EPA) and, as such, have proven safe for agricultural use. However, given the large number of atoxigenic isolates of *A. flavus* native to Kenya identified in this study, and similar diversity documented in West Africa (3), justification for such introductions remains questionable.

In addition to the processes described above, through which atoxigenic isolates may influence aflatoxin contamination of crops, the ability of a fungal isolate to persist within the local agroecosystems in the absence of a target crop is important. Such environmental fitness is particularly important for atoxigenic isolates being developed for low-income regions where farmers consume most of the crop and have limited economic incentive to apply aflatoxin mitigation measures. In these regions, responsibility may fall to public organizations to apply atoxigenic isolates to crops. Adaptation of atoxigenic isolates to the target agroecosystem favors the use of native atoxigenic isolates of *A. flavus* for reducing aflatoxin contamination of crops. Among the three VCGs of *A. flavus* studied to date, divergence appears to have occurred 18,000 to 63,000 years ago (37). Since this divergence, variation among VCGs has developed in diverse characteristics as isolates of the VCGs adapted to ecological niches. Isolates of native VCGs are adapted to compete for local resources and local cropping systems (including crop rotations and co-cropping). Native isolates should allow wider and more persistent displacement of aflatoxin-produc-

ing strains than exotic isolates. Native atoxigenic isolates are also genetic resources of the source countries (52), where the governments have the responsibility to manage such resources for maximum benefit of the population, as outlined in the Kenyan Environmental Management and Co-ordination Act of 2006 (52,61).

In this study, atoxigenic *A. flavus* isolates belonging to 12 different VCGs were found in multiple maize samples in the Eastern Province of Kenya. Recovery of an atoxigenic VCG from the target crop at multiple locations in a region potentially indicates a successful phenotype with competitive advantage in that agroecosystem. Isolates of such VCGs may be the best candidates for biocontrol products designed to provide long-term reductions in aflatoxin contamination. The number of VCGs detected within multiple samples in this study was surprising. A single atoxigenic isolate was collected from each of 37 of the 51 maize samples evaluated. Thus, only one VCG could be associated with most of the samples. This limited capacity to detect multiple VCGs within a sample These VCGs represent an important start for developing commercial biological control agents for maize aflatoxin contamination in Kenya. The large number of atoxigenic VCGs detected provides opportunity to utilize VCG mixtures similar to those developed for West Africa (2). Isolate mixtures may compete effectively in a greater diversity of environmental niches than individual isolates. Furthermore, modified *Aspergillus* communities with multiple atoxigenic VCGs are expected to have greater stability than those formed with single VCGs. This is important in regions where low-income farmers consume their crops directly, and where annual applications of a biological control product may not be feasible economically.

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