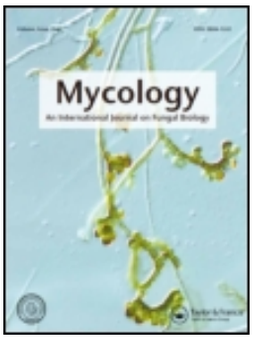

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What can the *Aspergillus flavus* genome offer to mycotoxin research?

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The genomic study of filamentous fungi has made significant advances in recent years, and the genomes of several species in the genus *Aspergillus* have been sequenced, including *Aspergillus flavus.* This ubiquitous mold is present as a saprobe in a wide range of agricultural and natural habits, and can function as an opportunistic animal and plant pathogen. *A. flavus* produces many secondary metabolites including aflatoxins, aflatrem and cyclopiazonic acid. In this chapter, our main focus is on the current status of the genomics of *A. flavus* as well as on the potential applications of genomics-based approaches to understanding mycotoxin production and fungal pathogenicity. It is hoped that the results of *A. flavus* genomics and functional genomics studies will empower researchers to find effective controlling strategies to eliminate mycotoxin contamination and to yield a safer and more abundant food and feed supply.

Keywords: *Aspergillus flavus*; mycotoxins; aflatoxins; secondary metabolites; gene clusters; fungal genomics

Introduction

Aspergillus flavus (teleomorph *Petromyces flavus*) is best known for its negative impact on agriculture. This common mold species not only produces many mycotoxins including the most toxic and most potent of all natural carcinogens, the aflatoxin family, but it also is an opportunistic pathogen for both plants and animals. For example, it can cause significant ear rot in corn. Under certain circumstances, *A. flavus* also can cause localized infections in humans and invasive aspergillosis in immunocompromised patients (Bennett 2009; Denning 1998).

Aspergillus flavus is one of the most abundant and widely distributed soil-borne molds in nature and can be found just about anywhere (Raper and Fennell 1965). It grows over a wide range of temperatures from 12 to 48 °C, with an optimal temperature range of 28–37 °C. The wide temperature range allows this mold to survive in climatic regions between latitudes 26° and 36° (Klich 2002). Its ability to grow at 37 °C facilitates its ability to serve as an opportunistic pathogen on humans and other warm blooded animals, while its metabolic versatility allows it to grow on many organic nutrient sources, including plant debris, decaying wood, animal fodder, compost piles, dead insect and animal carcasses, and stored grains (Denning et al. 1991; Denning 1998; Hedayati et al. 2007; Klich 1998). During its growth cycle, the fungus exists in the form of a rapidly growing mycelium. Reproduction occurs by the formation of asexual spores called conidia. Under certain adverse conditions, such as desiccation and poor nutrition, mycelia may congregate to form resistant structures called sclerotia, which may contribute to overwintering survival in agricultural settings. Sclerotia as well as conidiospores germinate under favorable growth conditions to produce new colonies (Raper and Fennell 1965).

Food safety

A. flavus not only reduces yields of agricultural crops through plant pathogenicity but it also decreases the quality of the harvested products through mycotoxin, especially aflatoxin, production. Corn, cotton, peanuts and tree nuts are the major crops that are regularly found contaminated with aflatoxins. Aflatoxin contamination in foods and feeds is an especially significant food safety issue in the developing world where the lack of an infrastructure for the detection, monitoring, and regulation of food contaminants means that hungry people often consume mycotoxin-containing food (Jelinek et al. 1989). It is estimated that approximately 4.5 billion people living in developing countries are chronically exposed to unknown amounts of aflatoxin that can have negative impacts on immune function and general health (Williams et al. 2004).

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Major outbreaks of acute aflatoxicosis in humans have been documented several times (Centers for Disease Control and Prevention 2004; Lewis et al. 2005). For example, in western India in 1974, 108 persons among 397 people affected died from aflatoxin poisoning (Krishnamachari et al. 1975). A more recent incident of aflatoxin poisoning occurred in Kenya in July 2004 leading to the death of 125 people among the 317 reported illnesses due to consumption of aflatoxin contaminated maize (Centers for Disease Control and Prevention 2004; Lewis et al. 2005). This kind of acute food poisoning problem is rarely observed in the US in humans, but does occasionally occur in animals. The most notable recent case involved the reported death of 23 dogs that had consumed aflatoxin-tainted dog feed as FDA and USA Today reported in December 2005 [http://www.foxnews.com/story/0,2933,180270,00.html; http://www.usatoday.com/news/nation/2005-12-30-dog-food_x.htm].

Acute aflatoxicosis is not the only concern. World health authorities caution that long-term dietary exposure to low doses of aflatoxins is also a major risk, leading to hepatocellular carcinoma (Bressac et al. 1991; Fung and Clark 2004; Hsu et al. 1991; Wogan 1992). The International Agency for Research on Cancer (IARC) has designated aflatoxin as a human liver carcinogen (Wogan 1992, 2000).

Because there is no effective control method to prevent aflatoxin contamination of food and feed, resulting in adverse effects in human and animals, the concentrations of aflatoxin in food and feed is carefully regulated in developed countries like the US (Eaton and Groopman 1994; Robens 2001; Robens and Cardwell 2005). Regulatory guidelines of the US Food and Drug Administration (FDA) set limits of 20 parts-per-billion (ppb) total aflatoxins for interstate commerce of food and feedstuff and 0.5 ppb aflatoxin M_1 in milk. Over 130 countries have established limits on the content of allowable concentrations of aflatoxin in food and feed. The European countries and Japan have set the most stringent levels. The European Commission has set the limits on groundnuts subject to further processing at 15 ppb for total aflatoxins and 8 ppb for aflatoxin B_1. For nuts and dried fruits subject to further processing, the maximum amount is set at 10 ppb for total aflatoxins and 5 ppb for B_1. The aflatoxin standards for cereals, dried fruits and nuts intended for direct human consumption are even more stringent, and the limit for total aflatoxins is 4 ppb and for B_1 is 2 ppb (Otsuki et al. 2001). Due to food safety regulations and monitoring, the threat of aflatoxin poisoning is low in developed countries. Nevertheless, the cost of keeping aflatoxin out of the food supply amounts to hundreds of millions of dollars to the US and world economy annually. In that sense, in developed countries, aflatoxin contamination is more of an economic issue than a food safety issue (Magan and Olsen 2004; van Egmond and Jonker 2005; van Egmond et al. 2007).

Historical overview of aflatoxin research

In reviewing the history of aflatoxin research, three major phases can be defined. The first milestone was the identification of aflatoxins as food poison in 1960 in conjunction with the occurrence of a mysterious "Turkey X" disease that killed more than 10,000 turkey poults in hatcheries in England. These poults had been fed with *A. flavus* infested peanut-meal (Allcroft et al. 1961; Asplin and Carnaghan 1961; Blount 1961; Lancaster et al. 1961; Nesbitt et al. 1962). After the toxic principle was isolated, it was named by abbreviating *Aspergillus flavus* toxin to *A-fla-toxin*. Mycologists discovered that in addition to *A. flavus*, *A. parasiticus* also produced aflatoxins. In most cases, *A. parasiticus* produced the four major aflatoxins: B_1, B_2, G_1 and G_2, while *A. flavus* produced only B_1 and B_2 (Kurtzman et al. 1987). The B-type toxins show blue fluorescence under long-wave UV light and the G-type toxins show green fluorescence (Davis et al. 1987). Finally, aflatoxin M_1 and M_2 are hydroxylated bioconversion products of B_1 and B_2, respectively, and were originally isolated from bovine milk (Garrido et al. 2003; Hsieh et al. 1985, 1986; Huang and Hsieh 1988; Rice and Hsieh 1982). *A. tamarii* (Goto et al. 1996), *A. nomius* (Kurtzman et al. 1987), *A. ochraceus*, and a few other species also produce aflatoxins under certain conditions (Cary and Ehrlich 2006; Ehrlich et al. 1999a; Kurtzman et al. 1987).

The second milestone was the discovery of a color mutant that accumulates brick-red pigment, norsolorinic acid (NOR), in *A. parasiticus* (Bennett et al. 1971, 1976, 1983; Bennett 1979). NOR is the earliest and the first stable aflatoxin precursor in the aflatoxin biosynthetic pathway (Bennett et al. 1997). This discovery opened the gate for the identification of a number of other aflatoxin intermediates (Bennett and Lee 1979; Bennett and Christensen 1983; Bennett and Papa 1988; Yao and Hsieh 1974) and then provided the opportunity to clone the first aflatoxin pathway gene, a reductase (Chang et al. 1992). Within a few years, several other important aflatoxin pathway genes were cloned, e.g. *aflD* (*nor-1*), *aflM* (*ver-1*), and *aflP* (*omtA*), *aflR*, and *aflB* (*fas-1*) (Chang et al. 1992; Keller et al. 1993; Skory et al. 1992, 1993) and a 70-kb aflatoxin pathway gene cluster was established in *A. parasiticus* and *A. flavus* (Yu et al. 1995). The discovery that nearly an entire fungal secondary metabolite pathway was encoded in a single gene cluster promoted renewed interest in aflatoxin biosynthesis by scientists all over the world. Significant progress has been made in elucidating this pathway, pathway intermediates, pathway genes, corresponding enzymes, and regulatory mechanisms (Townsend 1997). At least 23 enzymatic steps were characterized or proposed

to be involved in bioconversion of aflatoxin intermediates to aflatoxins. In fact, the aflatoxin biosynthetic pathway became one of the best studied fungal secondary metabolic pathways (Bhatnagar et al. 1992; Cai et al. 2008; Crawford et al. 2008; Yabe and Nakajima 2004). Comprehensive reviews on the complete aflatoxin pathway genes and gene clusters consisting of 30 genes in *A. parasiticus*, *A. flavus*, and *A. nidulans* were reported (Yu et al. 2004a,b, 2011).

The third milestone in aflatoxin research is the result of breakthroughs in high throughput DNA sequencing technologies that have revolutionized the biological sciences. Since the beginning of the millennium, the pace of genomics research has advanced dramatically. In turn, the development of high throughput genomic technologies has stimulated new approaches to data handling and processing. Not surprisingly, the power of genomics research has been brought to *A. flavus* and the aflatoxin problem. One important finding is that, when aligned with the genome sequence data, the aflatoxin gene cluster was located on chromosome III in a subtelomeric region (Yu et al. 2008, 2010b). However, the data generated and knowledge gained from the genomics research has far wider implications than an improved understanding of the structural genes involved in aflatoxin biosynthesis. Some examples of new insights from genomics research are described in the sections below.

In summary, during the 20 years from 1960 to 1980, most aflatoxin research was focused on the identification, detection, toxicological effects in animals and human beings, and primary control strategies. Simultaneously, the ground work for understanding the aflatoxin biosynthetic pathway was accomplished. The application of gene cloning during the early 1990s led to the elucidation of the aflatoxin gene cluster. At the writing of this chapter (2011), the third phase of aflatoxin research, fueled by insights from genomics, is in its infancy. This third phase of aflatoxin research is based on new synergies between scientists from disparate disciplines, including agriculture, biochemistry, computer science, genetics, systems biology and toxicology.

Aflatoxin biosynthesis, gene cluster and regulation

Aflatoxins are the best-studied mycotoxins. Biosynthetically, they are polyketide-derived secondary metabolites. Their structures are composed of bis-furan-containing dihydrofuranofuran and tetrahydrofuran moieties (rings) fused with a substituted coumarin. The aflatoxin pathway represents one of the best studied pathways of fungal secondary metabolism (Bennett and Goldblatt 1973; Bennett 1979; Bhatnagar et al. 2006; Chang et al. 1995; Cleveland and Bhatnagar 1990; Yu et al. 1993, 1998, 2004b). Aflatoxin biosynthesis involves numerous enzymatic reactions and structurally-defined aflatoxin intermediates. Genetic studies on aflatoxin biosynthesis in *A. flavus* and *A. parasiticus* led to the cloning and identification of 30 genes responsible for enzymatic conversions in the aflatoxin pathway that are found clustered within a 75-kb DNA region (Yu et al. 2004a, 2011). Many of the aflatoxin pathway genes and their corresponding enzymes have been characterized in both *A. flavus* and *A. parasiticus* (Yu et al. 2004a, 2010a, and references within).

The early aflatoxin biosynthesis pathway (from acetate to versicolorin B (VERB) or versicolorin A (VERA)) includes formation of several intermediates that are brick red, yellow or orange pigments (Bennett, 1979). The later aflatoxin pathway intermediates from VERB or VERA to the four aflatoxins are toxins which are colorless under normal light and which fluoresce under UV light. Norsolorinic acid (NOR), a red anthraquinone, is the first stable intermediate in the aflatoxin pathway (Bennett 1981; Bennett et al. 1997). VERB is a critical branch point leading either to B_1 and G_1 or to B_2 and G_2 formation. The two cytochrome P450 monooxygenases encoded by *aflQ* (*ordA*) (Yu et al. 1998) and *aflU* (*cypA*) (Ehrlich et al. 2004) are the two key enzymes for the formation of aflatoxin G_1 and aflatoxin G_2 in *A. parasiticus* and *A. flavus*.

A positive regulatory gene, *aflR*, genetically regulates both the aflatoxin and sterigmatocystin gene clusters. The *aflR* gene encodes a sequence-specific zinc binuclear DNA-binding protein and is required for transcriptional activation of most, if not all, of the aflatoxin pathway genes (Chang et al. 1995, 1999a,b; Ehrlich et al. 1999a,b; Woloshuk et al. 1994). Aflatoxin pathway gene transcription is activated when the AflR protein binds to the palindromic sequence 5′-TCGN5CGA-3′ (also called AflR binding motif) in the promoter region of these structural genes (Ehrlich et al. 1999a,b; Fernandes et al. 1998) in *A. parasiticus, A. flavus* and *A. nidulans*. *A. sojae* and *A. oryzae* are closely related nontoxigenic species used in industrial fermentation and were found to contain a defective AflR transcription activation domain due to the early termination of 62 amino acids from its C-terminus (Matsushima et al. 2001; Takahashi et al. 2002). The absence of the functional AflR regulatory protein means that no aflatoxin can be produced by these important food grade *Aspergillus* species. A co-regulatory gene, *aflS* (*aflJ*), divergently transcribed to the *aflR* gene in the aflatoxin gene cluster is also involved in transcriptional regulation (Meyers et al. 1998). The AflJ protein binds to the carboxy terminal region of AflR for transcriptional activation of the structural genes (Chang and Todd 2003). When *aflS* is disrupted in *A. flavus* no aflatoxin pathway metabolites are produced (Meyers et al. 1998). A global regulatory gene, *laeA* (named such for lack of *aflR* expression) was identified initially in *A. nidulans* (Bok and Keller 2004; Butchko et al. 1999). The *laeA gene* encodes a putative methyltransferase containing an *S*-adenosylmethionine (SAM) binding motif. A homologue of this regulatory gene, *laeA*, was also found in *A. flavus*

(EST ID: NAGEM53TV; AFLA_033290) (Bok and Keller 2004; Kim et al. 2006). Disruption of *laeA* resulted in loss not only of *aflR* gene expression for sterigmatocystin synthesis in *A. nidulans*, but also expression of genes involved in penicillin biosynthesis in *A. nidulans*, as well as genes involved in gliotoxin biosynthesis in *A. fumigatus* (AFUA_1G14660) (Bok and Keller 2004). Thus, *laeA* appears to be involved in the global regulation of a number of different secondary metabolic pathways in several fungal species. However, the exact mechanism by which it regulates secondary metabolite gene expression is not well understood.

Another interesting aspect of the mycotoxin story concerns a gene named *veA* that functions in light-dependent conidiation in *A. nidulans* (Mooney et al. 1990; Mooney and Yager 1990). Deletion of *veA* abolishes light dependence and results in the complete loss of ST and aflatoxin production under both light and dark conditions in *A. nidulans* and in *A. flavus* (Duran et al. 2007; Stinnett et al. 2007). Other studies have shown that *veA* modulates expression of *aflR* in *A. flavus, A. parasiticus* and *A. nidulans* (Calvo et al. 2004; Duran et al. 2007; Kato et al. 2003). The *veA* gene also was found to be necessary for the formation of sclerotia as well as to regulate the synthesis of cyclopiazonic acid and aflatrem. A *veA* deletion mutant of *A. flavus* did not produce aflatrem and showed greater than a two-fold decrease in cyclopiazonic acid production. Northern hybridization analysis showed that *veA* is required for expression of the *A. flavus* aflatrem genes *atmC*, *atmG* and *atmM* (Duran et al. 2007).

Aspergillus genomics

Genomics is the process of revealing the entire genetic content of an organism by high throughput sequencing of the DNA and bioinformatics identification of all of the genes. Genomics and its derivative fields include sequencing, annotation, gene identification, functional genomics, and the characterization of genes and gene products. Current genomic technologies allow scientists to sequence, annotate, and study whole genomes in a very short time with significantly reduced cost (Starkey and Elaswarapu 2010). The genomes of thousands of species from all life forms (viruses, bacteria, fungi, protista, and multicellular plants and animals) have been sequenced and annotated The technology itself has evolved from the basic automatic sequencing instruments to more and more high capacity technologies and platforms. We can foresee that even more efficient sequencing technologies will be on the market in a few years, in part because the National Human Genome Research Institute has put forth goals to reduce the cost of human genome sequencing to $100,000 in the short term, and $1000 in the longer term, to spur the innovative development of technologies that will permit the routine sequencing of human genomes for use as a diagnostic tool for disease (Hert et al. 2008). Already several instruments capable of producing millions of DNA sequence reads in a single run are available. These fast and low-cost DNA sequencing technologies include the Roche (454) GS FLX sequencer using pyrosequencing chemistry, Illumina Genome Analyzer using polymerase-based sequencing-by-synthesis, and Applied Biosystems SOLiD sequencer using ligation-based sequencing. The cost per reaction of DNA sequencing has fallen in accordance with Moore's Law (Moore 1965). The throughput has been doubled each year. These technologies change the landscape of genomics research and provide the ability to answer certain questions with previously unimaginable speed. They have broad applications ranging from chromatin immonoprecipitation, mutation mapping, polymorphism discovery, genome comparisons, non-coding RNA discovery, anti-sense RNA, small RNA discoveries, full-length cDNA analyses to serial analysis of gene expression (SAGE)-based methods. Next-generation sequencing has the potential to bring enormous changes in genetic and biological research and to enhance our fundamental biological knowledge. Unlike many microarray studies, RNA sequencing (RNA-Seq) can provide access to a cell's entire transcriptome with almost infinite resolution and can reveal additional levels of transcript complexity.

Members of the genus *Aspergillus* were among the first filamentous fungi to be studied by genomic methods (Bennett and Arnold 2001). In addition to the *A. flavus* genome (Payne et al. 2008), the genomes of several related *Aspergillus* species have been sequenced, including the *A. fumigatus* (Nierman et al. 2005), *Neosartorya ficheri* (anamorph *A. fisheri*; Fedorova et al. 2009), *A. oryzae* (Machida et al. 2005), *A. nidulans* (Galagan et al. 2005), *A. niger* (Baker 2006; Pel et al. 2007), *A. terreus*, and *A. clavatus* (Fedorova et al. 2009) projects (Table 1). Last year, a Community Sequencing Program to sequence eight *Aspergillus* species was started at the Joint Genome Institute (JGI). An additional 25 *Aspergillus* species are among the candidate list for sequencing by the *Aspergillus* community. These genome resources have been and will be available in the *Aspergillus* Genome Resources at http://www.ncbi.nlm.nih.gov/projects/genome/guide/aspergillus/index.html or http://www.fgsc.net/Aspergillus/asperghome.html. Two monographs that cover many aspects of recent *Aspergillus* research, including early genome-based studies, are available (Goldman and Osmani 2008; Machida and Gomi 2010).

The availability of these genome sequences will facilitate research on basic biology, infection mechanisms, host-fungus interactions, mycotoxin synthesis, genetic regulation, and evolution through comparative genomic studies of these closely related *Aspergillus* species. Genomic resources for *A. flavus* include ESTs, whole genome data, and microarrays that provide a platform for functional genomic studies. Beyond the identification of the structural

Table 1. *Aspergillus* genomes sequenced and under sequencing.

Aspergillus species	Strains	Genome size (Mb)	No. genes	PKS	%GC	Sequenced by
nidulans	FGSC-A4	30.07	10,560	41	50.32	Broad Institute
fumigatus	Af293	29.38	9887	30	49.8	TIGR/JCVI, Sanger
fumigatus	FGSC A1163	29.21	9906	30	49.8	Celera, Merk, TIGR/JCVI
N. fischeri	NRRL181 or 183	32.55	10,406	29	49.43	TIGR/JCVI
oryzae	RIB40, ATCC42149	37.12	12,063	37	48.24	NITE, AIST
flavus	NRRL3357	36.79	12,604	40	48.35	TIGR/JCVI, NCSU, ARS/SRRC
clavatus	NRRL1	27.86	9121	26	49.21	TIGR/JCVI
terreus	NIH2624	29.33	10,406	32	52.9	Broad Institute
niger	NRRL3,ATCC9029	33.7	14.000	41	50.36	Integrated genomics
niger	CBS 518.88	33.9	14,165	n/a	50.4	DSM
niger	NRRL328, ATCC1015	37.2	11,200	n/a	n/a	DOE JGI
aculeatus	CBS 172.66	n/a	n/a	n/a	n/a	DOE JGI
carbonarius		36.3	11,624	n/a	51	DOE JGI

genes involved in secondary metabolite biosynthesis, these genomic resources may guide the identification of virulence factors expressed during fungal infection of human, other animals, and plants. Moreover, genomics research promises to deliver new targets that can be screened for antifungal drugs; new insights for the breeding of crops resistant against fungal invasion; and for a broader identification of genes of cellulosic and other hydrolytic enzymes for waste recycling and for biofuel production.

Aspergillus flavus expressed sequence tags

The largest set of *Aspergillus flavus* ESTs was generated from a cDNA library of wild type strain NRRL 3357 (ATCC #20026) (Yu et al. 2004c). Over 26,110 cDNA clones from a normalized cDNA expression library were sequenced at The Institute for Genomic Research (TIGR, now named J. Craig Venter Institute, JCVI). Then, 19,618 *A. flavus* ESTs were generated, from which 7218 unique EST sequences were identified (Yu et al. 2004c). The 19,618 EST sequences are available at the NCBI GenBank Database (http://www.ncbi.nlm.nih.gov/) with accession numbers from CO133039 to CO152656. The *A. flavus* Gene Index also was constructed at TIGR and is currently maintained and curated by The Dana Farber Cancer Institute (http://compbio.dfci.harvard.edu/tgi). Using the EST database, four new transcripts (*hypB*, *hypC*, *hypD*, and *hypE*) were identified in the aflatoxin biosynthetic gene cluster. These transcripts had not been identified previously by chromosomal walking. In addition, several candidate genes possibly involved directly or indirectly in aflatoxin biosynthesis were identified, including genes for global regulation, signal transduction, pathogenicity, virulence, and fungal development (Yu et al. 2004c). The *A. flavus* ESTs were useful both for the construction of amplicon microarrays and the assembly of the genomes of both *A. oryzae* and *A. flavus* (Machida et al. 2005).

Aspergillus flavus whole genome sequencing

The *flavus* NRRL3357 whole genome sequencing project was funded by a USDA, National Research Initiative grant awarded to Professors Gary A. Payne and Ralph Dean, North Carolina State University, Raleigh, North Carolina. In addition, the Food and Feed Safety Research Unit of Southern Regional Research Center, USDA/ARS, New Orleans, Louisiana, provided funding for fine finishing and gene calling. The sequencing was completed at TIGR/ JCVI under the supervision of Dr. William C. Nierman, by a shotgun approach and Sanger sequencing protocol. There are many informative facets to the *A. flavus* genome story. At the most basic level, we now know that the *A. flavus* genome is organized into eight chromosomes with a genome size of about 36.8 Mb. Aided by the *A. flavus* EST database, the *A. oryzae* EST database, and the *A. oryzae* whole genome sequence, annotation of the sequence data postulates that there are about 12,604 genes in the *A. flavus* genome, a number similar to those of other sequenced *Aspergillus* species (Galagan et al. 2005; Machida et al. 2005; Nierman et al. 2005; Yu and Cleveland 2007) (Table 3). The availability of *A. flavus* and *A. oryzae* genome sequences allowed scientists to identify the genes for several major mycotoxins. Candidate genes putatively involved in the biosynthesis of secondary metabolism include those encoding polyketide synthases (PKSs,) non-ribosomal peptide synthethases (NRPS), cytochrome P450 monooxygenases, fatty acid synthases (FAS), carboxylases, dehydrogenases,

reductases, oxidases, oxidoreductases, epoxide hydrolases, oxygenases, and methyltransferases are identified (Payne et al. 2008; Yu et al. 2010). The sequence data have been deposited to the NCBI RefSeq database (http://www.ncbi.nlm.nih.gov) with the accession no. AAIH02000000, and are also available through the *Aspergillus flavus* website (http://www.aspergillusflavus.org), *Aspergillus* Comparative Database of The Broad Institute at MIT (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html), and Central Aspergillus Data Repository in the United Kingdom (http://www.cadre-genomes.org.uk/aspergillus_links.html).

Microarrays as tools for functional genomics studies

Microarrays are robust tools used for functional genomics studies. In recent years, several types of *A. flavus* microarrays have been constructed (Table 2). The first cDNA amplicon microarray, consisting of 753 gene features, including known aflatoxin pathway genes *aflD* (*nor-1*) and *aflP* (*omtA*) and regulatory gene *aflR*, was constructed at North Carolina State University. The unique ESTs identified from a cDNA library constructed using *A. flavus* RNA obtained under aflatoxin-producing condition were spotted on Telechem SuperAldehyde glass slide using an Affymetrix 417 Arrayer (O'Brian et al. 2003). The 5002 gene-element *A. flavus* amplicon microarray was constructed by the Food and Feed Safety Research Unit, USDA/ARS/SRRC. The amplicons were obtained by PCR using primers designed according to EST sequence information and template from genomic DNA. This microarray has been updated to a 5031 gene-element array including new genes of interest when their sequences became available. The Food and Feed Safety Research Unit,

Table 2. *Aspergillus flavus* microarrays constructed.

Array type	Genes included in the design	Genes represented	Probes (Amplicons or oligos)	Note
Amplicon array	Initial amplicons designed	5002	5002	Three times duplicated
Amplicon array	amplicons + 29 aflatoxin genes	5031	5031	Three times duplicated
70-mer oligo array	Annotated genes	11,820	11,869	
	aflRas	1	1	
	MAT2	1	1	
	oryzae unique genes	278	278	
	Corn genes resistant to *A. flavus*	10	10	
Total		12,125	12,159	Two times duplicated
Affymetrix array	Annotated *A. flavus* genes	12,834	13,115	
	Putative antisenses	397	397	
	Aflatoxin cluster genes	34	34	
	Aflatoxin cluster intergenic tilling	52	52	
	A. flavus PKSs	35	35	
	A. flavus NRPSs	24	24	
	Genes unique to *A. oryzae*	417	426	
	A. nidulans ST cluster genes	25	34	
	A. parasiticus	2	2	
	A. fumigatus	1	1	
	S. bicolor	1	1	
	F. graminearum tricothecene genes	22	41	
	F. verticillioides fumonisin genes	23	46	
	Corn genes	8400	8400	
	Human, mouse, *E. coli*, control	75	75	
Total		**22,343**	**22,683**	

Table 3. Fifty five secondary metabolism gene clusters predicted in *Aspergillus flavus.*

Cluster #	Backbone gene	Cluster gene locus	No. of genes in cluster
1	PKS	AFLA_002820-AFLA_002920	3
2	?	AFLA_004220-AFLA_004300	9
3	NRPS	AFLA_004440-AFLA_004450	2
4	NRPS	AFLA_005430-AFLA_005460	4
5	PKS	AFLA_006170-AFLA_006190	3
6	NRPS	AFLA_008700-AFLA_008810	12
7	NRPS/PKS	AFLA_009120-AFLA_009210	10
8	NRPS&PKS	AFLA_009980-AFLA_010060	9
9	NRPS	AFLA_010550-AFLA_010640	9
10	?	AFLA_016120-AFLA_016140	3
11	NRPS	AFLA_022880-AFLA_023090	12
12	NRPS	AFLA_028710-AFLA_028730	3
13	NRPS	AFLA_038600-AFLA_038630	4
14	?	AFLA_041040-AFLA_045540	8
15	?	AFLA_045490-AFLA_045540	2
16	PKS	AFLA_053780-AFLA_053870	9
17	NRPS	AFLA_053770-AFLA_053870	6
18	PKS	AFLA_054260-AFLA_054310	8
19	?	AFLA_060660-AFLA_060710	6
20	PKS	AFLA_062800-AFLA_062890	10
21	NRPS	AFLA_064240-AFLA_064610	38
22	NRPS	AFLA_066690-AFLA_066840	6
23	PKS	AFLA_066820-AFLA_066980	17
24	NRPS	AFLA_069320-AFLA_069340	3
25	NRPS	AFLA_070840-AFLA_070920	9
26	PKS	AFLA_079320-AFLA_079430	12
27	PKS	AFLA_082140-AFLA_082180	5
28	NRPS	AFLA_082450-AFLA_082490	5
29	?	AFLA_084080-AFLA_084090	2
30	NRPS	AFLA_090170-AFLA_090220	6
31	NRPS	AFLA_094980-AFLA_095120	25
32	?	AFLA_096250-AFLA_096460	10
33	NRPS/PKS	AFLA_096700-AFLA_096770	8
34	NRPS	AFLA_100280-AFLA_100350	8
35	NRPS	AFLA_101690-AFLA_101730	5
36	PKS	AFLA_104210-AFLA_104270	7
37	NRPS	AFLA_105170-AFLA_105190	3
38	PKS	AFLA_105440-AFLA_105460	4
39	PKS	AFLA_108540-AFLA_108580	5
40	PKS	AFLA_112820-AFLA_112900	9
41	PKS	AFLA_114800-AFLA_114820	3
42	PKS	AFLA_116160-AFLA_116260	11
43	PKS	AFLA_116560-AFLA_116660	19
44	PKS	AFLA_116870-AFLA_116920	6
45	PKS/NRPS	AFLA_118310-AFLA_118460	16
46	PKS	AFLA_118940-AFLA_118970	4
47	NRPS	AFLA_119090-AFLA_119140	6
48	NRPS	AFLA_121440-AFLA_121540	20
49	PKS	AFLA_125600-AFLA_125650	6
50	PKS	AFLA_126660-AFLA_126730	8
51	PKS	AFLA_127070-AFLA_127110	5
52	PKS	AFLA_128030-AFLA_128090	7
53	NRPS	AFLA_135430-AFLA_135500	13
54	PKS	AFLA_139140-AFLA_139410	30
55	PKS/NRPS	AFLA_139460-AFLA_139500	4

USDA/ARS/SRRC has also constructed a comprehensive whole genome *A. flavus* 70 mer oligonucleotide microarray. This whole genome microarray represents 11,820 *A. flavus* unique genes, the unique genes present in *A. oryzae* but absent in *A. flavus*, and 10 corn genes that show resistance against *A. flavus* infection. A whole genome *A. flavus*

Affymetrix GeneChip (microarray) funded by USDA/NRI contains elements representing all of the *A. flavus* genes, a subset of *A. oryzae* genes not found in *A. flavus* NRRL 3357, approximately 8400 genes expressed in corn seed, and a few genes from *Fusarium* species, and the mouse and human genomes. Additionally, a 6932 gene-element peanut 70 mer oligonucleotide microarray (which represents expressed peanut genes) based on 13,879 peanut EST sequence data, funded by Crop Protection and Management Laboratory, USDA/ARS, Tifton, Georgia, has been constructed at J. Craig Venter Institute (JCVI) for studies on plant-fungus interaction during fungal infections.

These *A. flavus* microarray resources provide a platform for functional genomics study on how genetic, environmental, and nutritional factors, influence aflatoxin production. Profiling of genes involved in aflatoxin formation using these microarrays, performed at USDA laboratories, the laboratories of North Carolina State University and JCVI has identified hundreds of genes that are significantly up or down regulated under various growth conditions (Chang et al. 2007; Kim et al. 2006; O'Brian et al. 2003, 2007; Price et al. 2005, 2006; Wilkinson et al. 2007a,b; Yu et al. 2007).

Almost all of the studies on aflatoxin biosynthesis in *A. flavus* and *A. parasiticus,* and on sterigmatocystin production *in A. nidulans* have focused on the 30 genes clustered within a 75-kb DNA region on a single chromosome (Yu et al. 2011). However, genes within the gene cluster do not account for all of the bioconversion steps of the aflatoxin pathway (Yu et al. 2004a,b). New approaches are necessary for identifying these other aflatoxin biosynthetic genes. It is hoped that gene profiling using microarrays, emerging next generation sequencing technologies and focused experimental manipulations will help identify additional structural and regulatory genes.

The regulatory mechanisms of aflatoxin formation have been investigated under diverse media and environmental conditions using *A. flavus* microarrays. Hundreds of candidate genes potentially involved in aflatoxin formation have been identified (Fedorova et al. 2009; Georgianna et al. 2010; Wilkinson et al. 2007a,b; Yu et al. 2007). However, due to the low copy number of regulatory genes and detection sensitivity of hybridization-based microarray detection, accurate detection of low abundance transcripts, which are common for regulatory genes, is difficult. It is possible that the subtle nature of genetic regulatory network of aflatoxin formation will become more obvious with new technologies, such as RNA-Seq, that recently became available for functional genomic analysis. For example, investigation in the expression patterns of aflatoxin and other mycotoxin genes under aflatoxin-producing (30 °C) and non-producing (37 °C) conditions revealed that high temperature negatively affects aflatoxin production by turning down transcription of the two key pathway regulators, *aflR* and *aflS*, with a more significant effect on *aflS* than on *aflR*. Change in the expression ratio of *aflS* to *aflR* from 4:1 to 0.7:1 rendered *aflR* nonfunctional for transcription activation (Yu et al. 2011).

Aspergillus flavus genomics in studying mycotoxins and other secondary metabolites

Background

It is well known that fungi have the capacity to produce a broad array of diverse secondary metabolites (Rank et al. 2010; Turner 1971; Turner and Aldridge 1983) and the genus *Aspergillus*, a member of the phylum *Ascomycota*, is a prolific producer of these low molecular weight compounds. Some of them are beneficial such as the antihypertensive drug lovastatin (Brakhage et al. 2008), but over 300 secondary metabolites produced by *Aspergillus* species are classified as mycotoxins (Cole and Cox 1981; Council for Agricultural Science and Technology 2003; Richard and Payne 2003). Depending on the concentration and mode of exposure, mycotoxins may have acute toxic effects leading to death or more difficult to diagnose illness from long-term exposure resulting in chronic effects, such as suppressed immune response, malnutrition, or induction of cancer (Hsieh 1989; Van Egmond 1989). The term mycotoxin is derived from the Greek words "MYKHE" (or "myco" meaning fungus) and "TO ELKON" (meaning arrow-poisons or toxins). Some mycotoxins are produced by a single species or by one specific strain; others are made by a number of species. Mycotoxins have very diverse chemical structures, diverse toxic effects and a variety of biological activities (Sweeney and Dobson 1998). Structurally, mycotoxins are synthesized from amino acids, shikimic acid or malonyl CoA through special pathways (Ehrlich 2007; Ehrlich et al. 1999a; Sweeney and Dobson 1999). *A. flavus* is notorious because it is the major producing species for aflatoxin, the most toxic and the most potent carcinogenic known mycotoxin. In addition, *A. flavus* also has the ability to produce aflatrem, cyclopiazonic acid (CPA), kojic acid, and several other toxic compounds as it colonizes pre- or post-harvest grains.

Aflatrem

Aflatrem is a tremorgenic mycotoxin that has profound effects on the central nervous system, causing tremors, mental confusion, seizures, and death (Gallagher and Wilson 1979; Valdes et al. 1985). Aflatrem also has been found to cause significant histopathological changes in the heart, liver and brain tissues of chicks (Rafiyuddin et al. 2006). The aflatrem biosynthetic pathway gene *atmG* from *A. flavus* was cloned using degenerate primers for conserved domains of fungal geranylgeranyl diphosphate synthase gene, *paxM*, from *P. paxilli*. Two other genes, *atmC* and *atmM* were identified adjacent to *atmG* (Zhang

et al. 2004). More recently, another gene *atmP* was identified as coding for a cytochrome P450 monooxygenase, which may be involved in aflatrem synthesis (Nicholson et al. 2009). A search of the complete genome sequence of *A. flavus* identified the presence of a putative aflatrem gene cluster, with the genes for aflatrem synthesis being split between two discrete regions (Nicholson, et al. 2009). The first cluster was found to be telomere-proximal on chromosome 5, while the second was telomere distal on chromosome 7 (Nicholson et al. 2009; Payne et al. 2008; Zhang, et al. 2004). Although the expression of aflatoxin biosynthetic genes correlated well with the onset of aflatrem production, low-level expression of genes in the telomere-distal region was detected prior to the onset of aflatrem synthesis. With the *A. flavus* EST and whole genome sequence data, it is predicted that at least eight genes are required for aflatrem biosynthesis (Zhang et al. 2004). *A. flavus* is the most widely described producer of aflatrem, but aflatrem also can be produced by *A. minisclerotigenes*, a species that resembles *A. flavus* in its ability to produce aflatoxins B_1, B_2, CPA, kojic and aspergillic acid (Pildain et al. 2008).

Cyclopiazonic acid (CPA)

CPA is an indole tetramic acid which inhibits sacroplasmic reticulum Ca^{2+}-ATPases by binding to calcium-free conformations of the ATPase enzyme (Soler et al. 1998) and blocking calcium channel access (Moncoq et al. 2007). Inhibition of these ATPases by CPA results in cell death as a result of stress-response and activation of apoptotic pathways (Venkatesh and Vairamuthu 2005; Vinokurova et al. 2007). In mice, CPA has an LD50 of approximately 13 mg/kg (Nishie et al. 1985) making consumption of a lethal dose unlikely, especially as there is no evidence that CPA accumulates in animal tissue. However, side-effects of low doses have not been ruled out as its pharmacological properties are similar to classical antipsychotic drugs such as reserpine and chlorpromazine. These side-effects include hyperkinesias, hypothermia, Parkinson-like tremors, catalepsy and convulsions (Nishie, et al. 1985).

CPA toxicity also has been linked to Turkey X disease. Turkey X disease was responsible for bringing *A. flavus* to notoriety with the discovery of aflatoxin. However, some of the symptoms of Turkey X disease are consistent with CPA poisoning and it is now hypothesized that Turkey X was the result of a synergistic effect of the simultaneous consumption of aflatoxin and CPA present in contaminated feed (Cole 1986; Richard 2008).

Recent studies on gene expression regulation of CPA and aflatoxins demonstrated that both toxins are produced in developing corn kernels during infection in the field, appearing first at 48 h after infection by the fungus. In the culture conditions tested, CPA was produced under conditions conducive as well as conditions non-conducive for aflatoxin production, indicating that its production may not be regulated the same way as that for aflatoxin (Georgianna et al. 2010).

After the release of the annotated genome sequence data, a cluster of CPA biosynthetic genes were identified and cloned in *A. flavus* (Chang et al. 2009) and *A. oryzae* (Tokuoka et al. 2008). A PKS-NRPS gene is necessary for CPA production (Tokuoka et al. 2008). Moreover, the predicted FAD oxidoreductase in CPA biosynthesis was described and named *maoA* (Chang et al. 2009). The genomic location of the CPA cluster was initially postulated (Chang, 2010, personal communication) to be close to the aflatoxin gene cluster as strains with partial deletions for the aflatoxin cluster failed to produce CPA. On the genomic chromosome physical map, the CPA biosynthetic genes were found to span a 20-kb DNA region near the telomere of chromosome 3, with only two predicted genes separating the aflatoxin cluster from the CPA cluster.

Kojic acid (KA)

Aspergillus flavus and *A. oryzae* both produce large amounts of kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone; KA) (Bentley 2006; Wilson 1971). KA was originally isolated from a koji culture, a solid-state culture of steamed rice inoculated with *A. oryzae* (Saito 1907). KA is widely used in pharmaceutical and fermentation industries. It can be used as an antibiotic, as an additive to prevent browning of food materials, and as an antioxidant (Bentley 2006). Recently, it has found use as a skin-lightning agent in the cosmetic industry and in medicine for the treatment of chloasma due to its inhibitory effect on tyrosinase (Cabanes et al. 1994; Saruno et al. 1979). Analysis of the genome sequences of *A. oryzae* (Machida et al. 2005), and *A. flavus* (Payne et al. 2006), and of gene expression data in *A. oryzae*, has identified and characterized two genes involved in KA biosynthesis (Terabayashi et al. 2010). One gene has a typical oxidoreductase motif and the other has a transporter motif.

Other mycotoxins

Bioinformatic analysis of the sequence data predicted a number of secondary metabolism gene clusters in the genome. Analysis using the software of Secondary Metabolite Unique Regions Finder (SMURF, http://jcvi.org/smurf/index.php) (Khaldi et al. 2010), led to the prediction of 55 secondary metabolism gene clusters (Table 3) (Fedorova et al. 2008, Georgianna, et al. 2010, Khaldi, et al. 2010). The predictive model identifies regions with a characteristic signature of genes known to be involved in secondary metabolism. The algorithm searches for multifunctional enzymes including nonribosomal peptide synthetases (NRPSs) for nonribosomal peptides, polyketide synthases (PKSs) for polyketides, hybrid NRPS-PKS

enzymes for hybrids, and prenyltransferases (PTRs) for terpenoids, all of which have been shown to be involved in secondary metabolism (Hoffmeister and Keller 2007; Keller et al. 2005). For example, the biosynthesis of aflatoxins requires a PKS, aflatrem requires a NRPS, and CPA biosynthesis involves a hybrid NRPS-PKS. Once the program identifies these putative multifunctional enzymes, it next searches the surrounding genes for motifs of genes associated with secondary metabolism. These data indicate that *A. flavus* has the potential to display a very diverse repertoire of secondary metabolites/mycotoxins.

Analysis of the gene expression data from the Affymetrix microarray experiments showed different gene expression patterns within the 55 secondary metabolism clusters predicted by SMURF. By hierarchy, they were classified into four discernable expression patterns (clades) (Georgianna et al. 2010). The aflatoxin pathway gene cluster, the CPA gene cluster and additional six other predicted clusters exhibited similar expression patterns and were placed into the same clade. This clade had moderate to high levels of gene expression and contained clusters that are predicted to encode other secondary metabolites such as pigments and siderophores.

Recently, the RNA-Seq technology was employed to characterize the *A. flavus* transcriptome at 30 and 37 °C and compare the expression patterns of the 55 predicted putative secondary metabolism gene clusters. Among these clusters, 11 were up-regulated at 30 °C compared with 37 °C (cluster #1, 11, 13, 23, 20, 21, 30, 43, 45, 54 and 55), while at 37 °C only two clusters were up-regulated (cluster #2 and 3) (Yu et al. 2011).

Despite these efforts, the biosynthetic pathways and gene products for most of the predicted secondary metabolites are still unknown. A good example is 3-nitropropionic acid (3-NPA), a rarely studied mycotoxin produced by *A. flavus*. Exposure to 3-NPA produces a range of toxicological effects and consumption of food contaminated with 3-NPA leads to health risks. Cases of acute poisoning are known. For example, 217 cases in was reported in China between 1972 and 1984 resulting in 88 deaths (Fu et al. 1995). 3-NPA irreversibly inactivates succinate dehydrogenase and impairs energy production within mitochondria (Johnson et al. 2000). Defects in mitochondrial energy productions play an important role in the pathology of neurodegenerative diseases (Luchowski et al. 2002). On the other hand, a safety assessment concluded that the risk associated with consumption of 3-NPA is small (Burdock et al. 2001). The genes for the biosynthesis of 3-NPA are yet to be identified, notwithstanding the availability of genome data.

In fact, among the 55 predicted clusters, only a few of the clusters were experimentally characterized for their biological functions. They are cluster #10 for conidial pigment, cluster #54 for aflatoxins, cluster #55 for CPA, and the spilt cluster for aflatrem (see above). The remaining uncharacterized clusters represent a pool of secondary metabolites yet to be discovered. It is hoped that analysis and comparison of several sequenced *Aspergillus* genomes will provide clues for identifying these unidentified gene products. Preliminary annotation has revealed that all of the sequenced *Aspergillus* genomes contain many backbone genes for secondary metabolism biosynthesis (Table 4). Uncharacterized secondary metabolite gene clusters could be coding for unidentified mycotoxins, compounds possessing pharmaceutical properties for drug development, or may represent unexpressed genetic relics. These compounds encompass pathway end-products, pathway intermediates, or shunt metabolites formed along these pathways. *A. flavus* secondary metabolite pathways may code for compounds with drug action. It is well known, for example, that *A. terreus* produces lovastatin, a potent cholesterol-lowering drug. Other aspergilli secrete antibiotics (penicillin and cephalosporin), antifungals (griseofulvin), and anti-tumor drugs (terrequinone A) (Hoffmeister and Keller 2007; Keller et al. 2005). Genomic resources and genomic research tools provide new opportunity to characterize secondary metabolism that may reveal pharmaceuticals active against fungal or bacterial pathogens. Nevertheless, even with accurate sequence data and good prediction of secondary metabolite gene clusters, we must not lose sight of the fact that identification of biological function requires experimental "wet lab" characterization.

Table 4. Predicted gene clusters for secondary metabolism in five *Aspergillus* species.

Species	PKSs	NRPSs	P450s
A. fumigatus	14	14	65
A. nidulans	28	14	102
A. oryzae	31	24	151
A. flavus	34	24	131
A. niger	34	17	?

Aspergillus flavus genomics for studying plant pathogenicity

A. flavus is an opportunistic pathogen of many agricultural crops, such as maize (corn), cotton, groundnuts (peanuts), as well as tree nuts, such as Brazil nuts, pecans, pistachio nuts and walnuts. Its ability to attack seeds of both monocots and dicots, and to infect seeds produced both above and below the ground, indicates that this fungus has evolved a remarkable battery of mechanisms to breach the host defense system. Compared to most fungal pathogens, *A. flavus* lacks host specificity (St Leger et al. 2000). It infects corn ears, cotton balls and peanut pods after insect or mechanical damages occur (Hocking 1997). Owing to its ability to grow at low water activity, *A. flavus* is also capable of colonizing seeds of grains and oil crops. Few plant

pathogenic fungi have such a broad host range. In general, high temperature, dry condition and plant stress are the environmental parameters that favor *A. flavus* infections in plants (Payne 1998).

A. flavus invasion of pre-harvest crops in the field is a complicated process involving multiple genetic and environmental factors (Brown et al. 2001; Cleveland et al. 2005). Only a few pathogenicity factors have been reported in *A. flavus*. The pectinase P2c, implicated in aggressive colonization of cotton bolls, is produced by most *A. flavus* isolates (Brown et al. 2001; Shieh et al. 1997). Invasion of cottonseeds has been associated with the production of a specific pectinase isozyme (Brown et al. 2001; Cleveland et al. 2005; Shieh et al. 1997; Whitehead et al. 1995). Lipases have also been described in *A. flavus* (Yu et al. 2003), but their role in pathogenicity is not well established. Hydrolytic activity of *A. flavus* plays an important role in absorbing nutrients from host plants for fungal growth. Hydrolytic enzymes, such as cellulases, glucanases, chitinases, amylases and pectinases, could be pathogenicity factors during fungal invasion of crops. The genes responsible for production of many of these enzymes are difficult to identify through conventional molecular cloning methods. However, the *A. flavus* EST and genome sequence databases have identified genes encoding a number of hydrolytic enzymes including amylase, cellulase, pectinases, proteases, chitinase, chitosanases, pectin methylesterases, endoglucanase C precursor, glucoamylase S1/S2 precursors, β–1,3-glucanase precursor, 1,4-β-D-glucan cellobiohydrolase A precursor, glycogen debranching enzyme and xyloglucan-specific endo-β-1,4-glucanase precursor.

Relatively little is known about crop–fungus interactions. Several compounds inhibitory to fungal growth have been isolated including chitinase, amylase and trypsin inhibitors (Brown et al. 2001; Chen et al. 1999; Cleveland et al. 2005; Fakhoury and Woloshuk 2001), and ribosome inactivating proteins (Nielsen et al. 2001). Fatty acid peroxides, known as oxylipins, affect aflatoxin formation (Wilson et al. 2001). With the availability of *A. flavus* whole genome microarrays, it is much easier to identify genes expressed during fungal invasion of crops. Genes involved in such processes could be targeted for inhibiting fungal growth and/or aflatoxin formation. Moreover, improved knowledge of the genetic basis of crop–fungus interactions could help plant breeders develop crops resistant to fungal infection (Cleveland et al. 2005; Guo et al. 2003).

Aspergillus flavus genomics for studying animal pathogenicity

A. flavus is the second leading cause of invasive and non-invasive aspergillosis in humans and animals (Denning et al. 1991; Denning 1998; Denning et al. 2003; Mori et al. 1998). "Aspergillosis" is a broad term used to describe a wide range of diseases caused by any of a number of the *Aspergillus* species. These diseases range from an "allergy"-type illness, allergic bronchopulmonary aspergillosis, to pulmonary aspergillosis, to a life-threatening generalized invasive infection. Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity disorder. It typically occurs in patients suffering from asthma or cystic fibrosis. Allergic fungal sinusitis is another allergic illness. In addition to *Aspergillus fumigatus* and *A. flavus*, *Aspergillus niger*, *Aspergillus clavatus*, *Aspergillus glaucus*, *Aspergillus nidulans, Aspergillus terreus*, *Aspergillus dustups*, *Aspergillus versicolor*, and a few other species are occasionally detected as pathogens in human and animal aspergillosis. Due to the increase of immunocompromised patients in the population (e.g. organ transplant, cancer therapies, and AIDS patients), the incidence of aspergillosis is rising (Denning 1998; Nierman et al. 2005; Ronning et al. 2005). In most cases, *A. flavus* causes severe illness only in immunocompromised individuals. The pathogen can attack any part of the body, from the skin to the sinuses, to the lungs, to the kidneys, and to the heart. Invasive aspergillosis is often fatal and there is a desperate need for better antifungal drugs to treat the increasing number of patients with various forms of aspergillosis.

A. flavus is often the most common cause of localized infections. Moreover, in geographical locations such as Saudi Arabia and Sudan, with arid and semi-arid weather conditions, invasive aspergillosis caused by *A. flavus* is more common than that caused by *A. fumigatus* (Kameswaran et al. 1992; Khairallah et al. 1992; Tierney et al. 1996; Yagi et al. 1999). In Saudi Arabia, among aspergillosis keratitis cases, *A. flavus* accounted for 80% of the total *Aspergillus* infections (Khairallah et al. 1992). In most other geographical locations, *A. fumigatus* is the more common causative agent. The high prevalence of *A. flavus*-associated infections may be due to the fact that the spores of this species can survive the hot and dry weather found in Sudan and Saudi Arabia. Nevertheless, serious *A. flavus* infections have been reported from other geographical locations; for example, of the human heart leading to endocarditis (Kennedy et al. 1998; Rao and Pediatr 2000) or pericarditis (Gökahmetoglu et al. 2000), and human eyes causing acute renal colic (Pérez-Arellano et al. 2001), and in the ear (Burkhart et al. 2000). In addition, *A. flavus* can be an insect pathogen (Kulshrestha and Pathak 1997).

Genes that contribute to *A. flavus* pathogenicity in human and animal infection must be expressed at mammalian and avian body temperature. Gene expression studies done under a series of temperature conditions identified hundreds of genes that are significantly over expressed at 37 °C compared to 30 °C (Yu et al. 2011). Further investigation by either gene knock-out or gene over-expression strategies will help to screen out the critical

genes responsible for thermotolerance (Nierman et al. 2005). Comparative genomic analysis of *A. flavus* versus *A. fumigatus* under those temperature conditions could help to identify the genes common in both aspergilli in response to temperature changes. The potential candidate genes include those encoding for heat shock proteins (HSP) and thermostable enzymes.

Aspergillus flavus genomics for economically useful processes

A. flavus is usually viewed in the context of its detrimental effects on human health as a pathogen and toxin producer. That said, mycologists have long recognized that *A. flavus* is closely related to *A. oryzae*, a species studied almost exclusively for its economically beneficial use in Asian food fermentations and as a major production species for industrial acids and enzymes. Now that genome data are available for both species, it is even more apparent that *A. flavus* and *A. oryzae* are genetically almost identical. However, *A. flavus* regularly is isolated from an extremely wide range of natural habitats while *A. oryzae* is a "domesticated" fungus, almost exclusively found in association with some form of human cultivation. In nature, *A. flavus* grows robustly on decaying vegetation, insect carcasses and other organic substrates. It is a wonderful recycler in the biosphere and, therefore, is likely to contain genes that could be harnessed for the economic conversion of biomass into fuel grade alcohols and hydrocarbons.

With improved genetic information from the genome project, genetic engineering could be used to remove the genes for mycotoxin formation or to add "good" genes to enhance the ability of *A. flavus* to degrade plant fibers and insect shells (e.g. by improving the expression of chitinase genes). *A. flavus* may be useful in carbon and nitrogen source recycling, particularly lignocellulose deconstruction, waste treatment, and energy regeneration. It may also have potential applications in industrial fermentations.

Future perspectives

Studies by traditional and molecular genetics techniques were successful in revealing that most of the steps of aflatoxin biosynthesis in *A. flavus* were encoded in a single gene cluster. A great deal remains to be learned, however, about the regulation of the aflatoxin pathway. It is hoped that high throughput genomic technologies will improve our understanding not only of aflatoxin biosynthesis but of many other aspects of fungal toxicology and mycotoxin production. Future genomic analysis will likely increase our understanding of the functional associations of genes and the way in which genome structure mediates the response of *Aspergillus* to its environment. It is hoped that we will be able to get a better understanding of gene function, genetic regulation, signal transduction, pathogenicity to humans and other animal as well as the fungus–host interaction during disease development. Future "omics" research will bring new insights in fundamental biology. For example, little is currently known about the role of microRNAs in *Aspergillus* gene expression. These small noncoding RNA genes are involved in gene silencing in many eukaryotic systems and may play a role in the differential expression of secondary metabolism in *A. oryzae* as compared to *A. flavus*.

In conclusion, genetic and genomic resources significantly enhance our ability to avoid the detrimental side and maximize beneficial effects of *A. flavus*. Taking a broad view of *Aspergillus* biology allows us to envision better ways to harness the remarkable genetic repertoire of this common mold.

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