

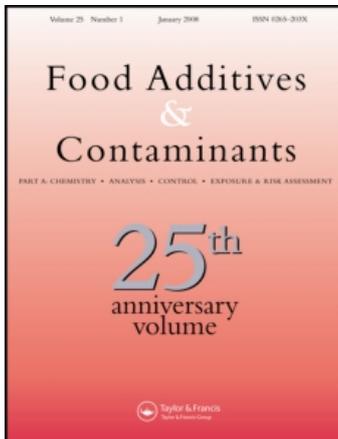
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Jiujiang Yu ^a; Gary A. Payne ^b; William C. Nierman ^{cd}; Masayuki Machida ^e; Joan W. Bennett ^f; Bruce C. Campbell ^g; Jane F. Robens ^h; Deepak Bhatnagar ^a; Ralph A. Dean ^b; Thomas E. Cleveland ^a

^a Southern Regional Research Center, USDA/ARS, New Orleans, LA 70124, USA ^b Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695, USA ^c The Institute for Genomic Research, Rockville, MD 20850, USA ^d Department of Biochemistry and Molecular Biology, The George Washington University School of Medicine, Washington, DC 20037, USA ^e National Institute of Advanced Industrial Science and Technologies (AIST), Tsukuba, Ibaraki 305-8566, Japan ^f School of Environmental and Biological Science, Rutgers University, New Brunswick, NJ 08901, USA ^g Western Regional Research Center, USDA/ARS, Albany, CA 94710, USA ^h USDA/ARS, Beltsville, MD 20705, USA

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Aspergillus flavus genomics as a tool for studying the mechanism of aflatoxin formation

Jiujiang Yu^{a*}†, Gary A. Payne^{b†}, William C. Nierman^{cd†}, Masayuki Machida^e, Joan W. Bennett^f, Bruce C. Campbell^g, Jane F. Robens^h, Deepak Bhatnagar^a, Ralph A. Dean^b and Thomas E. Cleveland^a

^aSouthern Regional Research Center, USDA/ARS, 1100 Robert E. Lee Blvd. New Orleans, LA 70124, USA; ^bDepartment of Plant Pathology, North Carolina State University, Raleigh, NC 27695, USA; ^cThe Institute for Genomic Research, 9712 Medical Center Drive, Rockville, MD 20850, USA; ^dDepartment of Biochemistry and Molecular Biology, The George Washington University School of Medicine, 2300 Eye Street NW, Washington, DC 20037, USA; ^eNational Institute of Advanced Industrial Science and Technologies (AIST), Central 6, 1-1, Higashi, Tsukuba, Ibaraki 305-8566, Japan; ^fSchool of Environmental and Biological Science, Rutgers University, 59 Dudley Road, New Brunswick, NJ 08901, USA; ^gWestern Regional Research Center, USDA/ARS, 800 Buchanan Street, Albany, CA 94710, USA; ^hUSDA/ARS, National Program Leader, 5601 Sunnyside Ave., Beltsville, MD 20705, USA

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Aspergillus flavus is a weak pathogen that infects plants, animals and humans. When it infects agricultural crops, however, it produces one of the most potent carcinogens known (aflatoxins). To devise strategies to control aflatoxin contamination of pre-harvest agricultural crops and post-harvest grains during storage, we launched the *A. flavus* genomics program. The major objective of this program is the identification of genes involved in aflatoxin biosynthesis and regulation, as well as in pathogenicity, to gain a better understanding of the mechanism of aflatoxin formation. The sequencing of *A. flavus* whole genome has been completed. Initial annotation of the sequence revealed that there are about 13,071 genes in the *A. flavus* genome. Genes which potentially encode for enzymes involved in secondary metabolite production in the *A. flavus* genome have been identified. Preliminary comparative genome analysis of *A. flavus* with *A. oryzae* is summarized here.

Keywords: aflatoxins; mycotoxins; toxigenic fungi; *Aspergillus*; genomics; expressed Sequence tags; microarrays; gene profiling

Introduction

Aflatoxins are natural secondary metabolites produced mainly by *Aspergillus flavus* and *A. parasiticus* (Bennett and Klich 2003), but *A. nomius*, *A. pseudotamarii*, *A. bombycis*, and several other species also have been identified as aflatoxin producers (Varga et al. 2003; Cary et al. 2005). *A. flavus* makes aflatoxins B₁ and B₂, while *A. parasiticus* produces aflatoxins B₁, B₂, G₁ and G₂ (Bennett and Klich 2003; Yu 2004). Aflatoxin B₁ is one of the most toxic and most potent hepatocarcinogenic natural compounds ever characterized (Williams et al. 2004). *A. flavus* infects a broad range of important agricultural crops, including both monocots and dicots (St Leger et al. 2000). It infects pre-harvest crops in the field, including maize ears, cotton balls, peanut pods and tree nuts, after insect or mechanical damage has occurred, as well as post-harvest grains during storage (Abnet 2007). *A. flavus* is often associated with contamination of aflatoxins (Cleveland et al. 2005) and renders crops un-consumable. Acute toxicity (Lewis et al. 2005) and long-term carcinogenicity (Wild and Turner 2002; Lewis et al. 2005) are correlated

with consumption of aflatoxin-contaminated food and feed in humans and animals. In Kenya, a recent outbreak of acute illness involved 317 cases and 125 deaths due to consumption of aflatoxin-contaminated maize (corn) (Lewis et al. 2005). Chronic dietary exposure to aflatoxins is a major risk of hepatocellular carcinoma in many third-world countries (Williams et al. 2004). *A. flavus* can also cause systematic disease in immunocompromised individuals. After *A. fumigatus*, *A. flavus* is the second leading cause of invasive and non-invasive aspergillosis (Denning et al. 1991; Yu et al. 2005).

Due to the health hazard of aflatoxins to humans and its adverse effects on livestock and livestock productivity, the aflatoxin content in food and feed is monitored and regulated in many countries of the world. The maximum allowable limit imposed by the United States Food and Drug Administration for consumption and interstate shipment of food and feed is 20 ng g⁻¹ (20 ppb). In the USA, the economic losses to farmers due to aflatoxin contamination of agricultural crops are estimated to be over \$270M

*Corresponding author. Email: jiujiang.yu@ars.usda.gov

†Yu, Payne and Nierman contributed equally to the *A. flavus* genome sequencing project and to this paper.

annually in years of severe outbreak (Robens 2001; Richard and Payne 2003; Robens and Cardwell 2005). In the developing world, aflatoxin detection and regulation are not stringently enforced due to a lack of adequate testing facilities. Consequently, the populations of these countries are exposed to aflatoxins by consumption of contaminated foodstuffs, and chronic exposure causes significantly higher rates of liver cancers (Williams et al. 2004). To minimize aflatoxin contamination of crops, food and feed, tremendous efforts have been made by scientists worldwide to better understand the toxicology of aflatoxins, biochemistry and genetic regulation of aflatoxin biosynthesis, as well as the biology, taxonomy and evolution of aflatoxigenic fungi.

Research on aflatoxins began with the identification of aflatoxins as toxic compounds in 1960 (Lancaster et al. 1961; Williams et al. 2004; Groopman et al. 2005). The first stable intermediate of the aflatoxin biosynthesis was identified less than a decade later (Bennett et al. 1971; Bennett et al. 1997). Molecular biological and molecular genetic studies on aflatoxin biosynthesis have been carried out within the last 15 years through gene cloning and genetic manipulations (Chang et al. 1992; Skory et al. 1992; Minto and Townsend 1997; Yabe and Nakajima 2004; Yu et al. 2004b). The generally accepted aflatoxin biosynthetic pathway has now been established (Cleveland and Bhatnagar 1990; Bhatnagar et al. 1992; Skory et al. 1992; Chang et al. 1993; Chang et al. 1995b; Bennett et al. 1997; Minto and Townsend 1997; Payne and Brown 1998; Ehrlich et al. 1999; Chang 2003; Yabe and Nakajima 2004; Yu et al. 2004b; Cleveland et al. 2005; Ehrlich et al. 2005; Yu et al. 2005). Genetic studies on aflatoxin biosynthesis in *A. flavus* and *A. parasiticus* led to the cloning of 29 genes responsible for enzymatic conversions in the aflatoxin pathway, all of which are clustered within a 82-kb DNA region (Yu et al. 1995; Yu et al. 2004a; Yu et al. 2004b). Many of the aflatoxin pathway genes and their corresponding enzymes have been characterized in both *A. flavus* and *A. parasiticus* (Yabe and Nakajima 2004; Yu 2004; Yu et al. 2004b). Several regulatory genes have also been cloned and characterized, namely *aflR*, required for transcriptional activation of most, if not all, of the structural genes (Chang et al. 1993; Payne et al. 1993), *aflS* (*aflJ*), involved in the regulation of transcription (Meyers et al. 1998; Chang 2003), and *laeA*, a global regulator involved in the regulation of numerous secondary metabolites in several fungal species (Butchko et al. 1999; Bok and Keller 2004).

Despite these significant advances, aflatoxin contamination problems are far from being solved. There remains a vast gap in our understanding of the coordinated global regulation of aflatoxin formation. Most importantly, no effective control strategies to

prevent aflatoxin accumulation have been developed. Genomics of the aflatoxin-producing fungus, *A. flavus*, will contribute to a better understanding of biosynthetic pathways, the mechanism of genetic regulation, the relationships between primary and secondary metabolism, the mechanism by which environmental conditions affect mycotoxin production, the field ecology, the mechanisms of fungal infection (fungus–host interactions) in plants, animals and humans, as well as the evolution of *A. flavus* as a crop pathogen and aflatoxin producer. This knowledge will, in turn, lead to biotechnological innovations and contribute to the development of new control strategies to minimize aflatoxin contamination, resulting in a safer, more economically viable food and feed supply.

We have successfully completed two *A. flavus* genomics projects: *A. flavus* Expressed Sequence Tags (EST) and genome sequencing and have used the resulting data to construct *A. flavus* microarrays. The *A. flavus* NRRL 3357 (ATCC # 200026; SRRC 167), a wild-type strain widely used in laboratory and field studies, was chosen for these genomics projects. *A. oryzae* strain RIB40 (National Research Institute of Brewing Stock Culture and ATCC-42149), a non-toxicogenic strain used in food fermentation industry in Japan for over a thousand years, and the *A. oryzae* genome sequencing strain (Machida et al. 2005), were used for comparative studies.

A. flavus sequence analysis

We employed an autoclosure technique on the sequenced genome to effectively close the sequencing gaps. Preliminary assembly of the raw sequence data resulted in 2080 contigs, which they were further assembled into a total of 79 scaffolds. The scaffold size ranged from 4.5 Mb to 1 kb. The 16 largest scaffolds may represent the 16 arms of the eight chromosomes of *A. flavus* genome. Initial annotation indicated that 99.6% of the predicted genes were within the 16 largest scaffolds. The *A. flavus* genome size is estimated to be about 36.8 Mb and contains about 13 071 predicted genes encoding for polypeptides that are equal to or larger than 100 amino acids.

A. flavus sequence comparison to *A. oryzae*

A. flavus has the ability to produce aflatoxins when it infects agricultural crops. It is also a pathogen that infects plants, animals and humans. Its close relative, *A. oryzae*, shares about 98% identity on the DNA level to *A. flavus*. *A. oryzae* is neither a pathogen nor does it produce such toxins, and is commonly used in the food fermentation industry, being granted GRAS status (Generally Regarded As Safe) by the United States Food and Drug Administration. The whole genome

sequence of *A. oryzae* was completed in Japan (Machida et al. 2005). The genomes of several other *Aspergillus* species, *A. fumigatus* (Nierman et al. 2005) and *A. nidulans* (Galagan et al. 2005) were also reported. The *A. flavus* genome is larger than either *A. nidulans* or *A. fumigatus*, but similar to its genetic cousin, *A. oryzae*.

We have begun a genome-wide comparison between *A. flavus* and *A. oryzae*. Our preliminary studies indicated that over 95% of the annotated genes are shared between the two species. Fewer than 300 genes are unique to each species. Special attention was paid to the structure of the aflatoxin pathway gene cluster in both *A. flavus* and *A. oryzae*. Structurally, the *A. oryzae* contains a roughly intact aflatoxin pathway gene cluster (Figure 1) (Watson et al. 1999). However, the *A. oryzae* cluster genes are not expressed due to a previously described defect in the *aflR* gene and an hypothesized, but as yet un-clarified, defect somewhere in the regulatory hierarchy (Kusumoto et al. 1998).

Identified genes of interest

Using classical genetic and biochemical approaches, we have identified 29 genes in a cluster in *A. flavus* and *A. parasiticus*. Most of these genes have been confirmed to be directly involved in aflatoxin biosynthesis (Yu et al. 2004b; Yu et al. 2004c). Preliminary annotation of the *A. flavus* genome revealed the existence of many genes that encode un-characterized polyketide synthases (PKS), fatty acid synthases, carboxylases, dehydrogenases, reductases, oxidases, oxidoreductases, epoxide hydrolases, mono- or dioxygenases, cytochrome P450 monooxygenases, methyltransferases (Chang et al. 1995a; Minto and Townsend 1997; Keller et al. 2000; Yu et al. 2004b), and non-ribosomal peptide synthases (NRPS). These genes could be involved in biosynthesis of other secondary metabolites in *A. flavus*. A transporter gene, *aflT* (Chang et al. 2004), has already been cloned from the aflatoxin pathway gene cluster. At first glance, we have seen as many as 77 ABC transporters in the *A. flavus* genome. These transporters could be involved in secondary metabolite secretion or in drug resistance. Other categories of genes potentially involved in secondary metabolite production are genes for global regulation, signal transduction, pathogenicity, virulence, oxidative stress responses (Mahoney et al. 2000; Mahoney and Molyneux 2004; Kim et al. 2005; Kim et al. 2006), and fungal development (Calvo et al. 2002). A homolog of the regulatory gene, *laeA* (Butchko et al. 1999; Bok and Keller 2004), has also been found in the genome annotation and is expressed in an *A. flavus* EST study (Yu et al. 2004c). The genome sequencing has been

completed but the genome is still undergoing annotation. A comprehensive gene list of categories in the *A. flavus* genome will soon be compiled and examined.

Genomic resources available

A total of 19,618 *A. flavus* ESTs were generated, from which 7218 unique EST sequences were assembled (Yu et al. 2004c). These ESTs were released to the public in NCBI GenBank database (<http://www.ncbi.nlm.nih.gov>) and the functional classification has been presented in Gene Index by The Institute for Genomic Research (TIGR, <http://www.tigr.org>; currently is managed by The Dana Farber Cancer Institute at the website <http://compbio.dfc.harvard.edu/tgi>). The *A. flavus* whole genome sequence data have been deposited to NCBI GenBank database (<http://www.ncbi.nlm.nih.gov>) and are also available through the *Aspergillus flavus* website (<http://www.aspergillusflavus.org>).

A 5031-gene element *A. flavus* microarray was constructed using the EST sequence information at TIGR and, subsequently, a number of gene profiling experiments have been performed with satisfactory results (Price et al. 2005; Price et al. 2006; Yu et al. 2006; Wilkinson et al. 2007). The *A. flavus* whole genome oligo and Affymetrix microarrays were designed and constructed at TIGR and by Affymetrix Inc. (Emeryville, Ca, USA), respectively. These arrays contained all of the *A. flavus* genes, *A. oryzae* unique genes, plus additional genes of interest from corn, *Fusarium* spp., mouse and human genomes. They are ready to be used for genome-wide gene expression profiling and for comparative genome hybridization.

Future prospects

The availability of the *A. flavus* genome sequence data will facilitate research on basic biology, infection mechanisms, host–fungus interactions, genetic regulation of mycotoxin synthesis, and the evolution of these *Aspergillus* species. Genes for many other important mycotoxins produced by *A. flavus*, such as cyclopiazonic acid (CPA), aflatrem and aspergillic acid, have not yet been identified and their biological functions remain unclear. Preliminary analysis of the *A. flavus* genome reveals an abundance of novel secondary metabolic genes or clusters of genes that are candidates for the biosynthesis of these mycotoxins. Moreover, the availability of *A. flavus* microarrays provides the opportunity to evaluate gene transcription during aflatoxin biosynthesis (O' Brian et al. 2003; Price et al. 2005; Price et al. 2006; Yu et al. 2006; Wilkinson et al. 2007) and fungal infection under specific conditions. The genome-wide gene profiling and

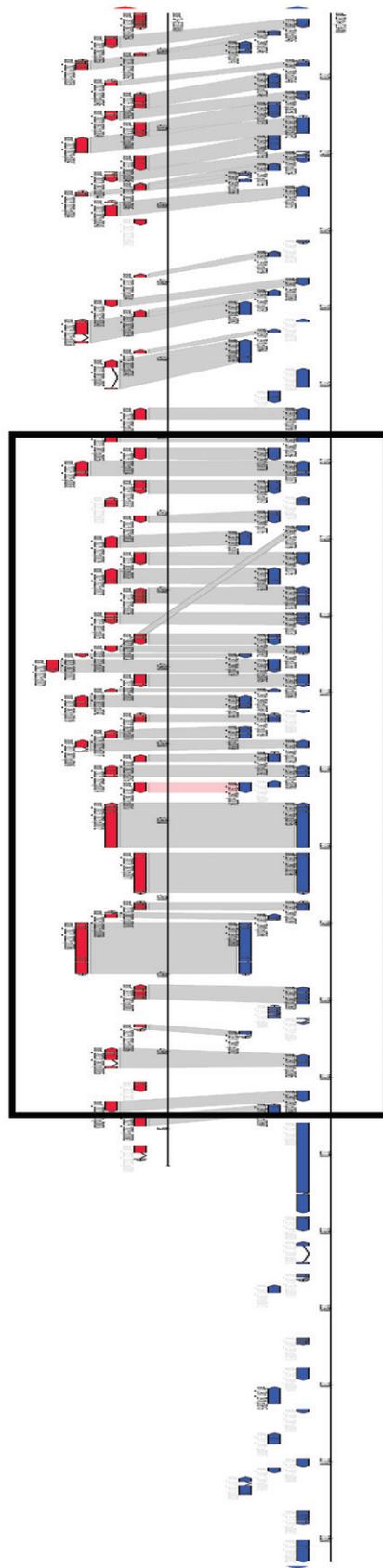


Figure 1. Comparison of aflatoxin pathway gene cluster region between *A. flavus* and *A. oryzae*. The 82-kb aflatoxin pathway gene cluster is shown within the box. The coding regions of the aflatoxin pathway cluster genes of *A. flavus* NRRL 3357 are represented by the dark horizontal bars on top and those of *A. oryzae* RIB40 are represented by the dark horizontal bars on bottom. The corresponding genes are aligned by the vertical or slanted bars. Arrows indicate the direction of gene transcription. This image is from a comparative gene model visual presentation using a SYBIL software program, which is developed by the J. Craig Venter Institute (JCVI).

comparative genome hybridization, using the newly constructed microarrays, are expected to provide robust tools in our research for better understanding the mechanism of aflatoxin biosynthesis. These genomic resources promise a bright future for the discovery of new antifungal drugs, for the breeding of crops resistant against fungal invasion, for the development of innovative strategies to prevent and cure diseases of humans, animals and plants, and for the minimization of aflatoxins in the food and feed. In addition, some beneficial features of *A. flavus* could be exploited; for example, *A. flavus* is an effective recycler in the biosphere. Therefore, *A. flavus* may be harnessed in the future for biofuel production, waste treatment and other useful applications.

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