ABSTRACT

PRICE, MICHAEL SCOTT. Classical and Modern Genetic Approaches Reveal New Gene Associations with Aflatoxin Biosynthesis in Aspergillus parasiticus and A. flavus. (Under the Direction of Gary A. Payne)

Production of aflatoxin (AF) in Aspergillus species is a highly regulated process involving transcriptional and post-transcriptional controls. Most of the regulation of AF production is focused through the pathway-specific transcriptional regulator aflR. While much is understood about the steps involved in biosynthesis, less is known about the regulatory circuits controlling AF production. A targeted cDNA microarray consisting of 768 genes was developed to investigate the effect of nitrogen source, carbon source, culture temperature and culture pH on AF production in A. parasiticus. Seventeen genes were identified as consistently differentially expressed with respect to AF, including three of the AF pathway structural genes. One of these genes, CA747470 was consistently downregulated with AF and was shown to repress AF production when overexpressed in A. flavus.

Using an expanded cDNA microarray consisting of 5002 genes from an EST sequencing project (USDA-ARS, SRRC), we investigated the impact of aflR deletion on the transcriptome of A. parasiticus. In addition to the AF pathway genes, five additional genes were found to be regulated by aflR: niiA, hlyC, hypA, nadA, and hypB. These additional genes all possess putative consensus binding sites for AflR. The expression data from this study was also compared to the previous targeted array study by looking at expression of 324 genes shared by both microarrays. Expression profiles for the AF genes present on both arrays were consistent between experiments. CA747470 was shown to be highly expressed in all conditions. Overexpression of CA747470 resulted in increased radial growth and decreased AF production.

Finally, a putative Rho-GDP dissociation inhibitor (Afrdi1) was deleted in A. flavus that was found to share a transcription profile with aflR with respect to AF. The Afrdi1 deletion strain exhibited repressed AF production, as well as a severe growth defect on minimal medium. The deletion mutant was phenotypically similar to the bem4
deletion strain of *S. cerevisiae*. The implication of this gene in AF regulation provides a direct link between vegetative growth and secondary metabolism in *A. flavus*.

This work provides insight into the regulatory networks responsible for regulation of AF production in *Aspergillus* species, and indicates where future investigations are needed to understand the biology of this important mycotoxin.
CLASSICAL AND MODERN GENETIC APPROACHES REVEAL NEW GENE ASSOCIATIONS WITH AFLATOXIN BIOSYNTHESIS IN *ASPERGILLUS PARASITICUS* AND *A. FLAVUS*

by

Michael Scott Price

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Department of Plant Pathology

Raleigh

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APPROVED BY:

_________________________________________  _______________________________________
Gary A. Payne                                            Ralph A. Dean
Chair of Advisory Committee

_________________________________________  _______________________________________
Margaret E. Daub                                          Ross W. Whetten
DEDICATION

The work contained in this thesis is dedicated to my daughter, Rachael. She was born the last year of my doctoral program. She reminds me every day just how precious the time we have here is, and makes me want to leave this world better than when she found it. I love you, Peanut.
BIOGRAPHY

Michael Scott Price was born in Wilmington, Delaware on December 22, 1973, at 5:52 PM (Just for you, Mom.). He attended New Castle Baptist Academy from kindergarten until graduating high school in 1991. After graduation he entered the University of Delaware Honors Program, majoring in Animal Science and minoring in Biology. While at UD, he also entered the Degree with Distinction program, and did research for a Senior Thesis under the direction of Dr. John Dohms. His thesis work was completed in May, 1995, when he graduated from UD with a B. S. in Agriculture.

Upon graduation from the University of Delaware, Michael entered the Microbiology Department at Colorado State University. After realizing this program wasn’t for him, he left and returned home to take a year off before entering the Plant Pathology Department at North Carolina State University. His Master’s thesis work, under the direction of Gary Payne, investigated the removal of copper and zinc from swine effluent, a project which endeared him to all in Gardner Hall. Especially when the effluent was autoclaved.

With functional genomics looming on the horizon, Michael decided to stay with the Payne Lab to pursue his doctoral degree. Again majoring in Plant Pathology, this time Michael minored in Functional Genomics, and utilized cDNA microarrays to investigate the regulation of aflatoxin biosynthesis. During his doctoral program, his knowledge of genetics allowed he and his wife to produce a lovely daughter, Rachael, who was born on August 10, 2004. With the close of his doctoral thesis work, Michael plans to continue his education by entering the Molecular Mycology and Pathogenesis Training Program, a postdoctoral research program funded by NIH. His future plans are to study the regulation of pathogenesis in fungi.
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Many thanks are due the Extremophiles Lab, headed by Dr. Robert Kelly. Their expertise in the manufacturing, hybridization, and analysis of microarrays were instrumental in ensuring the quality of the data presented herein. In particular, I wish to thank Matt Johnson, Clemente Montero, and Shannon Conners for their patience and assistance. They were able to accomplish in months what would have taken me years on my own. Thanks to their hard work in fine-tuning the whole process, I am now able to assist others in obtaining array data in TWO WEEKS (whereas my first arrays took 2 YEARS; that seems so unfair!).

Certainly I wish to thank the many members of the Payne Lab whom I have known over the years, and a few in particular. Kirsten Nielsen was very helpful in honing my debating skills (No, Kirsten, that’s not what the data means…). Her friendship during the last years of her PhD really helped smooth out some rough spots in mine. Kim Scheidegger (Schwartzburg) was and continues to be a good friend. We helped each other work through the wonderful world of microarrays (…but, they’re so EASY…). Lastly, Greg OBrian also deserves my thanks for our many discussions on everything from God to the merits of bacon cream cheese on bagels.

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Last, but certainly not least, I wish to thank my family for their support and help in so many areas. My mother- and father-in-law have both been a tremendous help to my wife and I during my graduate education, and words can’t express how grateful I am to
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Genetics and gene regulation of the aflatoxin biosynthetic pathway.*

Michael S. Price and Gary A. Payne

Department of Plant Pathology, North Carolina State University, Raleigh, NC, U. S. A.

INTRODUCTION

Fungi are both a blessing and a curse to mankind. On the one hand, they have proven useful for millennia in the production of foods and other products. Indeed, the history of their use goes back at least as far as the production of wine and ale. However, they have also been responsible for some of the most insidious ailments we have faced. The process of ergot alkaloid contamination of grains by *Claviceps purpurea*, leading to the disease known as St. Antony’s Fire, is one prime example of the importance of mycotoxins in human health. Disease caused by this toxin was recorded as far back as the Middle Ages, and was probably responsible for such traumatic social reactions as the Salem Witch Trials [1].

In our current age, the prospect of weaponizing microbes or their products is especially horrifying. Biological weapons have been fashioned using mainly bacteria and viruses as human or animal pathogens. Similarly, there has been interest in fungi not as pathogens, but for the toxins they may produce. It has been documented that T-2 toxin (from *Fusarium spp.*) and aflatoxin (from *Aspergillus spp.*) have been produced by Iraq and other countries for use in weapons programs [2, 3].

Aflatoxin (AF) is a regulated mycotoxin that can occur in almost any poorly stored commodity or prepared food. It may also be produced in corn, peanuts, tree nuts and cotton prior to harvest. Interstate trade in the US is not permitted for food and feed containing greater than 20 ppb aflatoxin B1, the most toxic aflatoxin species. The allowable level in milk is less that 0.5 ppb (FDA). Many other countries have lower allowable levels of aflatoxin concentration (CAST, 2003). Contamination of corn by aflatoxin-producing fungi can be a problem in years when the summers are dry and hot, conditions that favor the growth of the aflatoxin-producing fungi *A. flavus* and *A. parasiticus*. While both of these species can contaminate crops, *A. flavus* is the most common of these pathogens on host crops pre-harvest, and constitutes most of the aflatoxin contamination problem [4].

Due to these concerns over food safety and human health, it would seem logical that careful study of the AF pathway should be a research priority. A full understanding of the biochemistry and genetics of the pathway may yield new control strategies for abolition of AF contamination of food crops. To date, much has been discovered about the biochemistry
of the AF pathway. Studies on a related pathway, the sterigmatocystin (ST) pathway in *A. nidulans*, have been instrumental in elucidating some of the genetics of AF/ST production. While the basic biochemical steps of the pathway are known and several regulatory factors have been identified [5-9], details of the molecular regulation of AF/ST production are complex and yet to be fully resolved. There have been numerous reviews covering the biosynthesis and ecological significance of this important secondary metabolite [10-16]. This review will cover the basics of the AF/ST pathway, and will attempt to illuminate some of the molecular mechanisms behind AF/ST regulation.

**BIOCHEMISTRY**

**Acetate to Hexanoic Acid**

The first stable intermediate in the aflatoxin pathway is synthesized from acetate and malonyl units by the concerted efforts of a fatty acid synthase (FAS) and a polyketide synthase (PKS) (Figure 1). The FAS synthesizes hexanoate, which is then elongated by the PKS through the addition of acetate units. The genes for both of these enzymes reside in the aflatoxin biosynthetic cluster. Evidence for hexanoate as the starter unit for aflatoxin biosynthesis was first provided by Townsend and colleagues [17], who showed that hexanoate provided to cultures could be converted into aflatoxin. Molecular studies support this hypothesis.

The gene identified as responsible for this assembly is *fas-1* [18]. Mahanti and colleagues performed UV mutagenesis on an *A. parasiticus* strain that accumulates norsolorinic acid (NA). Mutants were selected for the inability to accumulate NA. One of these mutants was then transformed with a cosmid that complemented both mutations, allowing AF production. Subsequent experiments led to the cloning of *fas-1*, which was shown to act upstream of the NA accumulation mutation.

Supporting biochemical evidence for *fas-1* encoding a hexanoate intermediate was obtained by Watanabe and co-workers [19]. Feeding of hexanoylNAC to a *fas-1* disruptant yielded low quantities of NA. The researchers speculated that Fas-1 and PKS must be
Figure 1. The aflatoxin/sterigmatocystin biosynthetic pathway. Gene names given in italics, with the genes from *A. nidulans* in parentheses. All other gene names are for *A. flavus/A. parasiticus*. 
associated for efficient conversion of hexanoate to noranthrone. Research in *A. nidulans* has also shed light on the relationship between primary and secondary metabolism. Two independent FAS complexes were described by Brown et al. [20], one which is required for primary growth (FAS), and another which is distinct to ST biosynthesis (sFAS). In *A. nidulans*, there are two sFAS genes in the ST cluster: *stcJ* and *stcK*. They showed that ST production was inhibited in disruptants of the sFAS genes, but could be restored with the addition of hexanoic acid. Disruptants of the primary FAS genes *fasA* and *fasB* were able to produce ST when supplemented with long chain fatty acids to allow for primary growth.

**Hexanoic Acid to Norsolorinic Acid**

Hexanoate appears to serve as the substrate for the aflatoxin polyketide synthase (Figure 1). Two PKS genes were cloned independently in *A. parasiticus*: *pksA* and *pksL* [21, 22]. *pksA* was identified based on sequence similarity to the *A. nidulans* *wa* gene, a PKS involved in spore pigmentation [23]. These two proteins each possess b-ketoacyl synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains, yet WA also has a thioesterase (TE) domain, which PksA lacks. Disruption of *pksA* in *A. parasiticus* led to inability of the fungus to produce aflatoxin and AF pathway intermediates, but showed no effect on spore color [21]. Feng and Leonard [22] found that disruption of *pksL1* in *A. parasiticus* also yielded loss of AF and AF pathway intermediate production. In fact, *pksA* and *pksL1* are the same gene. BLAST analysis of the two genes reveals they are identical, as was suggested in a previous review [15].

PKS activity leads to the production of noranthrone, presumably from a hexanoate starter unit [17]. The first stable intermediate in the aflatoxin pathway, however, is norsolorinic acid (NA; Figure 1). It is as yet unknown how the conversion from noranthrone to norsolorinic acid occurs, and there are three competing hypotheses. The conversion may occur spontaneously, as put forth by Dutton [10]. Vederas and Nakashima [24], proposed that a noranthrone oxidase could be responsible for this conversion. To date, no such enzyme has been found in aflatoxigenic fungi. Others have proposed that a monooxygenase may be responsible [25]. This may be the most likely scenario as there are two known
monooxygenases found in the ST biosynthetic cluster which have no assigned function at present: *stcB* and *stcW*.

**Norsolorinic Acid to Versicolorin B**

The steps from NA to versicolorin B (VERB) are fairly well established, with the only real controversy existing at the first step from NA to averantin (AVN; Figure 1). Two genes have been identified with the capacity to carry out the reduction of NA to AVN: *nor-1* and *norA*. *nor-1* was the first aflatoxin biosynthetic gene cloned, and was able to complement the NA accumulation phenotype in *A. parasiticus* [26]. Computer analysis of the gene revealed a putative peptide of 29 kDa, and indicated the peptide would be a ketoreductase [27]. Recently, Zhou and Linz [28] purified recombinant Nor1 protein using an *E. coli* expression system and confirmed the ketoreductase activity of this enzyme.

The second ketoreductase gene found in the AF cluster is *norA*. The protein encoded by this gene was isolated and shown to possess ketoreductase activity [29]. Monoclonal antibodies were raised against this purified protein and used to screen a cDNA library to isolate the *norA* gene. Computational analysis of the open reading frame encoded by *norA* showed similarity to the *Phanerochaete chrysosporium* aryl-alcohol dehydrogenase gene. The relationship of these two enzymes within the AF pathway is unclear. It is known that NA-accumulating mutants of both *A. flavus* and *A. parasiticus* still produce low levels of aflatoxin [30-34]. Perhaps the seeming redundancy at this step in the pathway alludes to the fecundity of norsolorinic acid production, or a need for the fungus to rapidly synthesize aflatoxin at the appropriate time (Figure 1).

The next step in the pathway involves the conversion of AVN to 5’-hydroxyaverantin (HAVN) by *avnA*. Bennett *et al.* [35] were first to place averantin in the aflatoxin biosynthetic pathway through the use of 14C-labeled averantin in feeding studies. The gene responsible for the AVN – HAVN conversion was first described by Yu and colleagues and given the designation *ord-1* when transcripts were identified using DNA sequence flanking the *omtA* pathway gene as a probe [36]. Sequencing and analysis of the cDNA clone corresponding to the *ord-1* transcript revealed homology to cytochrome P-450 enzymes, in
particular monooxygenases and dehydrogenases. Disruption mutants of ord-1 fail to produce aflatoxin, and accumulate a yellow pigment identical to averantin [37]. Metabolite feeding studies using norsolorinic acid or averantin failed to support aflatoxin production, whereas 5’-hydroxyaverantin, averufannin, averufin, versicolorin A, sterigmatocystin and O-methylsterigmatocystin supported aflatoxin production. avnA shows high similarity to the stcF gene found in the A. nidulans sterigmatocystin biosynthetic cluster, a gene linked to this same conversion [38].

Recently adhA, a gene encoding a putative alcohol dehydrogenase, was implicated in the conversion of 5’-hydroxyaverantin to averufin [39]. This gene, found within the AF biosynthetic cluster, exhibits coordinated expression with other known pathway genes when the fungus is grown under AF conducive conditions. Disruption of adhA in A. parasiticus leads to an accumulation of 5’-hydroxyaverantin and a small amount of O-methylsterigmatocystin. This is most likely due to a second, minor route of averufin formation through an averufannin intermediate (Figure 1). The authors [39] postulated that AdhA may oxidize HAVN to 5’-ketoaverantin (KAQN), which could then spontaneously cyclize to form AVF. This route, utilizing AdhA, is probably the major route in the pathway as evidenced by the phenotype of the adhA disruptant.

The conversion of AVF to versiconal hemiacetal acetate (VHA) has been hypothesized to include multiple enzymatic reactions [25]. To date, only one enzyme has been characterized as responsible for this conversion [40]. Using an AVF-accumulating mutant, Yu and colleagues were able to determine that the avfA gene product from A. parasiticus was responsible, at least in part, for the conversion of AVF to VHA. Transformation of the avfA gene into the AVF-accumulation strain complemented the phenotype to restore AF production. Computational analysis suggested that the stcO gene in A. nidulans may be the homolog of avfA. These researchers were also able to recover avfA homologs from A. flavus and A. sojae. It is still unknown whether this gene is solely responsible for the AVF-VHA conversion.

Nothing is currently known about the conversion from VHA to versiconal (VAL; Figure 1). This step involves removal of an acetaldehyde group, but no enzyme has yet been
demonstrated as responsible for this process. Versicolorin B synthase (vbs) has been shown to produce versicolorin B (VERB) from either enantiomers of VAL [41]. This is an extremely important step in the synthesis of AF as the bisfuran ring, which is responsible for the DNA binding property of aflatoxin, is formed. vbs resides in the AF gene cluster approximately 3.3 kbp upstream of omtA, [36, 42]. An open reading frame approximately 1400 bp long separates the two genes, which putatively encodes a cytochrome P-450 monooxygenase. However, no function has been experimentally determined for this putative gene.

**Versicolorin B to Aflatoxin**

Versicolorin B represents a major branch point in the AF pathway (Figure 1). The two major aflatoxins, B1 and B2, are delineated at this step. AFB1 is the more carcinogenic of the two, and is suspected to be a major contributing factor for liver cancer in humans [43, 44]. VERB is converted to versicolorin A (VERA), which eventually leads to AFB1 production. stcL, a putative cytochrome P-450 monooxygenase, has been shown to be important in the VERB to VERA conversion in the *A. nidulans* ST pathway [45]. Disruptants fed exogenous VERA were able to produce both ST and DHST (dihydrosterigmatocystin), whereas those not fed exogenous VERA could only produce DHST. This enzyme putatively carries out the desaturation of the bisfuran moiety in VERB to yield VERA, but this has not been experimentally proven. Furthermore, the homolog of *stcL* has not yet been identified in AF producing species.

VERB can also be used as a substrate for another enzyme to produce demethyl-dihydrosterigmatocystin (DMDHST). This reaction, as well as the VERA to demethylsterigmatocystin (DMST) conversion, have been hypothesized to require up to four different enzymatic steps including ketoreduction, oxidation, decarboxylation and methylation [25]. One gene shown in *A. nidulans* to be partly responsible for this conversion is *stcU*. *stcU* encodes a putative ketoreductase, and has been demonstrated as necessary for the conversion of VERB to ST and DHST in a stcL background [45]. The homolog of *stcU* in *A. parasiticus* is *ver1* [46]. Two copies of *ver1* exist in *A. parasiticus*: *ver1A* and *ver1B*
The second copy of this gene, \textit{ver1B}, was recently demonstrated to encode a truncated, inactive protein \cite{48}. \textit{ver1B} is located elsewhere in the genome of the fungus, away from the AF cluster, in an apparently duplicated AF cluster region \cite{48}.

The conversion from VERB/VERA to DMDHST/DMST also requires the protein product of \textit{stcS}. This gene in \textit{A. nidulans}, first named \textit{verB} \cite{49}, encodes a putative cytochrome P-450 monooxygenase and has been hypothesized as responsible for the oxidation component of this conversion \cite{50}. \textit{stcS} disruptants failed to produce ST under conducive conditions, and was shown in metabolite feeding studies to be involved at this step. However, Keller and colleagues \cite{50} were unable to demonstrate oxidation capability for this protein using cross-feeding experiments with a \textit{stcU} disruptant. No new data have been published for \textit{stcS} as of this review.

The next two reactions in the AF biosynthetic pathway involve the addition of methyl groups \cite{51}. Only one of these reactions occurs in \textit{A. nidulans}, as ST is the final product in this fungus. \textit{stcP} is the gene identified as responsible for the conversion of DMST to ST in \textit{A. nidulans} \cite{52}. Kelkar and colleagues demonstrated that a \textit{stcP} disruptant of \textit{A. nidulans} accumulated demethylsterigmatocystin and produced no ST. \textit{stcP} transcripts had been previously shown to be co-regulated with other ST pathway gene transcripts \cite{53}. Homologs of \textit{stcP} have been found in \textit{A. oryzae} (\textit{dmtA}), \textit{A. parasiticus} (\textit{dmtA} or \textit{omtB}), \textit{A. flavus} (\textit{omtB}) and \textit{A. sojae} (\textit{omtB}) \cite{54, 40}; in no case has the gene been disrupted.

The second methylation presumably occurs as the result of the activity of the putative methyltransferase \textit{omtA} (previously \textit{omt-1}), driving the conversions of ST/DHST to O-methylsterigmatocystin (OMST)/dihydro-O-methylsterigmatocystin (DHOMST) \cite{55, 56}. Antisera raised against the 40 kDa protein isolated by Keller \textit{et al.} \cite{55} was used to isolate a cDNA clone from an \textit{A. parasiticus} expression library. Upon further analysis, recombinant protein expressed in \textit{E. coli} was capable of converting ST to OMST \cite{56}. However, \textit{omtA} remains to be analyzed by gene disruption.

The final step in the AF pathway leading to the production of aflatoxin-B1 (AFB1) and AFB2 from OMST and DHOMST respectively is catalyzed by \textit{ord-1}. Prieto and colleagues \cite{57} first identified this gene utilizing cosmid constructs containing different
segments of the AF gene cluster. They were able to show that one construct conferred the ability to convert OMST to AFB1 on A. flavus strain 649, which is a mutant lacking the entire AF cluster. Further analysis narrowed the placement of ord-1 to a 3.3 kb region of the cosmid. Subsequently, Prieto and Woloshuk [58] were able to identify the coding sequence of ord-1. This gene codes for a putative cytochrome P-450 monooxygenase which is responsible for this final oxidoreduction step. Ord-1 protein was expressed in S. cerevisiae and demonstrated to convert exogenously fed OMST to AFB1.

**REGULATION**

The regulation of aflatoxin biosynthesis has been studied since 1965, when the role of kojic acid was investigated [59]. Since that time, four major nutritional and environmental stimuli affecting aflatoxin production have been characterized: nitrogen source, carbon source, temperature and pH. Additionally, development plays a major role, and may be an overriding factor in aflatoxin production. Indeed, the regulation of this mycotoxin appears to be highly complex (Figure 2).

The AF/ST biosynthetic pathway is under the direct control of the pathway specific transcriptional regulator, aflR [60-64]. Another gene which shares a 737 bp stretch of DNA as a promoter region with aflR and is divergently transcribed is aflJ [65]. The exact function of this gene is unknown. Meyers *et al.* [65] did show that if aflJ is disrupted no aflatoxin is produced, yet the pathway genes are transcribed under AF conducive conditions. Further, the aflJ open reading frame does not appear to encode a transcription factor as does aflR. The predicted peptide exhibits three membrane spanning motifs and a peroxisomal targeting signal [65].

It is also possible that aflJ is regulated by the global nitrogen regulatory gene areA. This possibility was first alluded to in a study focused on aflR overexpression [66]. While determining the effects of aflR overexpression on nitrate inhibition, a slight inhibition of aflJ transcription by nitrate was demonstrated. While not quantitative, the decrease in transcription seen on a Northern blot is noticeable. A later study by Ehrlich and Cotty [67]
Figure 2. Representation of the regulatory apparatus impacting aflatoxin biosynthesis.

The major inputs affecting the activity of the aflatoxin pathway specific regulator *aflR* are shown, including developmental (ie. FadA), nutritional, (ie. AreA) and environmental (ie. PacC) regulators. An unknown temperature response element (TRE) is inferred from the effect of temperature on the transcriptional and post-transcriptional control of AflR.

showed that the effect of nitrate on expression of *aflJ* is dependent on the genotypic background in which the experiment is conducted. These researchers demonstrated that *S_B* strains of *A. flavus* were much less inhibited by nitrate than were *S_BG* strains of the fungus. They also showed that transcript levels of *aflJ* were induced 2.6-fold in the *S_B* background, whereas they were repressed 2-fold in the *S_BG* background. The authors speculated that this difference in response to nitrate may be due to the amount and placement of AreA consensus binding sites in the *aflJ/aflR* intergenic region.

**Nutritional and Environmental Factors**

Aflatoxin is a complex carbon-containing molecule, and as such it is reasonable to assume that certain carbon sources would be better substrates than others. Indeed, much research has been done that has identified supportive and inhibitive carbon sources [68-72].
Generally, simple sources of carbon such as glucose are supportive for aflatoxin production. More complex sources of carbon, such as peptone, lactose and oleic acid do not support aflatoxin production. The genetics of carbon source regulation have not yet been discovered.

Recently, a gene cluster was identified in *A. parasiticus* which is adjacent to the AF pathway [73]. This cluster of four genes includes a putative regulatory gene (*sugR*), a putative NADH oxidase (*nadA*), a putative glucosidase (*glcA*), and a gene with homology to hexose transporters (*hxtA*). Yu and colleagues were able to show correlated expression of the *hxtA* gene with aflatoxin biosynthetic genes under conditions conducive for AF production. It is possible that this cluster is involved with the carbon regulation seen in AF producing fungi, but as of yet no conclusive data have been shown.

Nitrogen source is a known influential factor for AF production. In general, organic sources support and inorganic sources do not support AF biosynthesis. Sources of nitrogen especially favorable to AF production are aspartate, glutamate, alanine, glutamine, proline, and ammonium sulfate [74-78].

The effect of nitrogen source on aflatoxin production is profound, but not absolute. Kachholtz and Demain [5] found that inorganic nitrogen sources, such as sodium nitrate, exhibited significant inhibition of averufin production in *A. parasiticus*. Sodium nitrate was the most inhibitory nitrogen source they tested, yet the fungus was still able to produce 65.9% of wild type averufin levels on a mg/g dry weight scale. Further, these researchers found no effect of nitrogen source when fungal mycelia were resuspended in media containing either NH₄ or NO₃ as the nitrogen source [5]. One possible explanation for the nitrate effect involves NADPH consumption in the cell. NADPH is a common cofactor involved in enzyme reactions. It has been speculated that the mannitol cycle is upregulated in the presence of nitrate as the nitrogen source, and that this diverts NADPH away from AF pathway enzymes leading to their inactivity [79]. Preliminary data from our lab do not support this hypothesis, as media amended with 100 µM NADPH was unable to ameliorate nitrate repression of aflatoxin biosynthesis (unpublished results).

Molecular evidence further supports the direct regulation of aflatoxin biosynthesis by nitrogen. The GATA type transcription factor *areA* (*Neurospora crassa* homolog *nit2*) is the
global transcriptional nitrogen regulator in *A. nidulans* [80]. This transcription factor has recently been isolated and characterized in *A. parasiticus* [81]. Chang and colleagues used electrophoretic mobility assays to show that AreA protein could bind GATA elements found in the 737 bp intergenic region between *aflR* and *aflI*, suggesting a role in the regulation of one or both of these genes. The intergenic region between AF pathway genes *nor1* and *pksA* has also been investigated in *A. parasiticus* [64]. In this study, many potential regulatory elements were discovered, including putative binding sites for AflR and the nitrogen regulatory genes AreA and NirA (the pathway specific nitrogen regulatory gene in *A. nidulans*). Furthermore, at least one AreA binding site was found in this region in three other AF producing fungi: *A. flavus, A. nomius*, and *A. pseudotamarii*. However, there was no AreA site found in the one AF producing isolate of *A. bombycis*.

In some cases, there appears to be cooperation between factors repressive to aflatoxin biosynthesis. Katchholz and Demain [5], as well as studies in our lab (unpublished results) have shown nitrate inhibition in the absence of increasing pH. Flaherty and Payne [66] demonstrated that nitrate inhibition of aflatoxin biosynthesis was due to a more complex phenomenon than simply repression of *aflR* transcription. Indeed, a strain of *A. flavus* expressing AflR constitutively was still unable to produce aflatoxin in media containing NO$_3$ as the nitrogen source. The pathway gene *omtA* was expressed identically regardless of the nitrogen source used, but aflatoxin production was virtually non-existent.

An association between aflatoxin repression by NO$_3$ uptake and increased pH has been demonstrated [82]. Cotty showed that aflatoxin production decreased and number of sclerotia formed increased as pH increased in the presence of nitrate. The opposite was true for ammonia. However, when the ammonia cultures were buffered to maintain a pH above 4.4, sclerotia production was unaffected but aflatoxin levels dropped almost four-fold from the NH$_4$-unbuffered levels.

Keller *et al.* [7] were able to show that increased pH is repressive to AF pathway gene transcription in its own right. In these studies, *A. nidulans* and *A. parasiticus* were grown under permissive nitrogen conditions, but the media were buffered in a pH range from 4-8. Transcripts of *stcU* and *ver1* were visualized by Northern blot using RNA harvested from
tissue grown at either pH 4, 5, 6, 7 or 8. Above pH 5, stcU transcripts were non-existent; the same was true for ver1 transcripts above pH 6. Production of AF and ST in these fungi paralleled the Northern blot data, dropping steadily as the culture pH rose.

Keller and colleagues data also link pacC, the global pH regulatory gene found in A. nidulans, to AF/ST pathway regulation. PacC is translated as an inactive pro-protein when the fungus is grown at acidic pH. As the ambient pH becomes more alkaline, the PacC pro-protein is proteolytically cleaved to yield the active transcription factor. This regulatory protein represses transcription of acidic-expressed genes and induces transcription of alkaline-expressed genes [83]. Keller et al. [7] found that stcU gene expression increased from 24-48 hours in a wild type background, but decreased over the same period in a pacC constitutive expression mutant. Furthermore, ST production also was lower as compared to wild type by as much as nine-fold.

The action of pacC expression on AF/ST biosynthesis is probably direct, as this transcription factor usually represses acid-expressed genes under alkaline conditions and AF/ST production is suppressed under these conditions. A putative PacC binding site has been reported in the promoter region of aflR, and has been shown to bind protein extracts from AF-induced mycelia of A. parasiticus [84]. While not conclusive proof, mutating the putative PacC binding site eliminated protein binding in the mobility shift assay performed by these researchers.

Temperature is also known to play a role in aflatoxin production, but the nature of the control it exerts on aflatoxin is still debated. Much early work was done to define the conducive temperatures for AF production [85-89]. Currently it appears that temperature influences aflatoxin biosynthesis by affecting the transcription of pathway genes [90, 8, 91]. Aflatoxin and sterigmatocystin production, in A. parasiticus and A. nidulans respectively, are regulated in opposite manners. In A. parasiticus, AF production was maximal at 27°C, and no AF was detected at 37°C [8]. Conversely, ST was generously produced at 37°C, and only small amounts were produced at 27°C. Feng and Leonard demonstrated that pathway genes, including pksA, aflR, stcA, stcE, and stcD, were not transcribed at the non-permissive temperature in each species, which argues for transcriptional control of AF/ST biosynthesis.
These data are in contrast to those by Liu and Chu [91], who saw low level expression of aflR at restrictive temperatures in A. parasiticus and A. nidulans, and lower AflR accumulation with no accumulation of the late-pathway protein OmtA at restrictive temperatures. This is consistent with the finding that AflR is under post-transcriptional control by PkaA, a protein kinase responsible for regulation of development and secondary metabolism in A. nidulans [92]. Perhaps transcriptional profiling will assist in determining the identity of these (post)transcriptional regulatory elements (TRE; Figure 2).

**Influence of Development**

Fungal development and secondary metabolism are inextricably linked. Current knowledge points to a heterotrimeric G-protein coupled receptor as the starting point for a signaling cascade leading to conidiation and secondary metabolite production [93, 94]. Understanding of the link between the two may yield improvements in human and animal health through the disconnection of fungal development and mycotoxin production.

The link between secondary metabolite production and development wasn’t established until recently, when Hicks and colleagues showed that a G-protein signal was involved in both processes [6]. This research involved molecular characterization of a series of “fluffy” mutants, so named because of their colony morphology due to a lack of conidiation [95]. Of the six loci/genes initially described as having the “fluffy” phenotype, two were found by Hicks and colleagues to act in a regulatory capacity: fluG and flbA (Figure 2). Mutations in these two genes resulted in loss of both conidiation and sterigmatocystin production. fluG encodes a putative enzymatic protein believed to be responsible for producing an extracellular signal for development [96]. Later work by this group demonstrated that overexpression of fluG in submerged culture lead to conidiation but not sterigmatocystin production [97], suggesting other factors are involved.

Loss of function mutations in flbA share the fluG mutant phenotype. In addition, cultures with this mutation exhibit autolysis after a few days [6]. Interestingly, FlbA has been linked to G-protein signaling, and contains a regulator of G-protein signaling domain [98]. FlbA interacts with FadA, a G-protein a subunit which represses development and
secondary metabolism in *A. nidulans* [99, 6]. Maintenance of vegetative growth is possible through FadA-GTP association. Once the GTPase activity of FadA is engaged, presumably through some action by FlbA, the fungus is permitted to continue development, and conidiation and secondary metabolism commences [6].

Other genes have been isolated from this G-protein signaling pathway in recent years. A cAMP protein kinase cascade has been inferred to interact with this system through the discovery of *pkaA*, a cAMP-dependent protein kinase which is believed to inhibit the pathway specific transcription factor AflR via phosphorylation [94] (Figure 2). More recent evidence may also point to another gene, *leaA*, which may mediate the repression by *pkaA* [94]. *leaA* appears to be negatively regulated by PkaA and AflR. Further work must be done to link these early steps with the AF/ST pathway.

**NEW TOOLS FOR DISCOVERY**

**Genomics Tools**

Recent advances in the field of molecular biology have lead to an explosion of information. The new field of genomics is leading to breakthroughs in agriculture and medicine by allowing the study of cellular processes on a holistic level. Whereas regulatory pathways were traditionally studied one gene at-a-time, we can now view the entire transcriptional complement of a cell at once [100, 101]. By combining 2D gel electrophoresis and mass spectrometry, we can know if those transcripts are being translated into protein. Advances in mass spectrometry are also making possible the elucidation of the entire metabolite profile of an organism. Clearly, these are exciting times.

Of these three main genomics technologies, transcriptional profiling (e.g. “microarrays”) will prove the most useful in exposing the regulatory networks involved in AF/ST production. Indeed, cDNA microarray technology has already been used to this end in other systems. Transcriptional profiles have been used to more accurately define cancer cell types by exposing the transcriptional programs inherent to those cell types [102].
Traditional methods of classifying lymphomas often group cancers into types that cannot be further divided due to irreproducibility of observations between diagnosticians.

Alizadeh and colleagues used a specialized microarray developed in-house (the “Lymphochip”; [103] to analyze various B-cell lymphomas and determine distinct transcriptional profiles for them. Using 96 normal and malignant cell types to generate 1.8 million data points, the researchers were able to resolve distinct sub-classes of the diffuse large B-cell lymphoma cancer type. This was done by building a compendium of 128 array experiments using the 96 cell types. Hierarchical clustering of the data revealed common expression patterns of subsets of the 17,856 elements on the array, leading to distinct transcriptional fingerprints for the various cancer types.

Transcriptional profiling has also been used to reveal regulatory networks in *Saccharomyces cerevisiae*. When using a microarray representing all the open reading frames of yeast, DeRisi and colleagues found genes which changed in their expression pattern with time, as the fungus shifted from anaerobic to aerobic metabolism [104]. Of particular interest were those genes whose transcription profile changed in the same manner as genes known to be involved in fermentation and respiration. Greater than 400 genes of unknown function were discovered which fit this scenario, giving the first glimpse as to their function.

New methodologies utilizing cDNA microarray technology show even more promise for determining unknown gene function. In particular, a database (or compendium) of expression profiles can be utilized to narrow the focus of research to a few genes. Using 300 different growth conditions, including different nutritional and environmental cues, and various mutants, a compendium of expression profiles was created in an attempt to elucidate the function of unknown genes in *S. cerevisiae* [105]. Using a compendium approach, microarray fluorescence intensity values were clustered within tissue types to reveal similarly expressed genes. The tissue types were then clustered based on expression pattern. Using this approach in yeast, Hughes and colleagues were able to define the functions of eight previously undefined genes, and also discover a new drug target [105]. Perhaps application
of this approach to the study of AF/ST pathway regulation will likewise illuminate the function of heretofore undefined genes.

**Metabolic Pathway Analysis**

Another approach that holds promise for further understanding the complex biochemistry of AF/ST biosynthesis is metabolic pathway analysis (MPA). This type of “genomic” analysis traces its theoretical roots to the 1980’s, and involves analyzing the stoichiometric properties of metabolic networks. While MPA is powerful for gaining insights into metabolic pathway regulation, it is highly mathematical in nature and beyond the scope of this review. Here, the overriding ideas behind MPA will be discussed and an example of using this type of analysis in studying primary metabolic pathways will be given. For more in-depth coverage of this topic, please see [106, 107].

From the biologist’s perspective, MPA is basically involved with measuring the amount of metabolite flux through a system [106]. This property of metabolic pathways can be modeled mathematically, using the formula $S \cdot v = 0$, where $S$ is a stoichiometric matrix made up of two vectors $m$ and $n$, which correspond to the number of metabolites and reactions in the network respectively. The vector $v$ refers to the speed or activity of each reaction, $n$. Because of the nature of this design, the model is scaleable, and could potentially encompass the entire metabolic network of a cell.

This kind of analysis has been used to study the metabolic control of the glycolytic pathway in *Trypanosoma brucei*, *Leishmania donovani* and *Trichomonas vaginalis*. [108]. Various genes involved in glucose metabolism, such as glyceraldehyde phosphate dehydrogenase (GAPDH) and phosphoglucose isomerase (PGI), and housekeeping genes such as $\beta$-tubulin were analyzed by using a gene regulation model: $1 = \rho_h + \rho_m$. Here $\rho_h$ equals the change in enzyme concentration divided by the change in flux, and $\rho_m$ equals the change in enzyme rate divided by the change in concentration of surrounding metabolites, multiplied by the change in concentration of surrounding metabolites divided by the change in flux. $\rho_m$ is summed over all metabolites that change. Northern blot analysis was performed on all the genes being analyzed, to track their expression levels throughout the
experiment. Also, the various constituents of the model were measured, such as protein levels and enzymatic activities.

According to ter Kuile and Westerhoff, some genes in the glucose pathway are mostly metabolically regulated. This was determined by plotting glucose consumption against enzyme activity, and comparing the results to the northern blots. For example, glycerol-3-phosphate dehydrogenase showed no increase in enzyme activity over nearly three orders of magnitude increase of glucose consumption, even though expression levels of the gene changed. This was also the case for PGI. Some genes showed a mix of transcriptional and metabolic regulation, such as GAPDH. In this case, enzyme activity was unchanged over one order of magnitude of glucose consumption. When the system reached a critical point between two and three orders of magnitude, enzyme activity increased, as did the transcriptional component of the regulation.

ter Kuile and Westerhoff did note that mRNA levels for the genes studied did not correlate with enzyme activities, suggesting there can be differences in the conclusions drawn by analyzing a network with MPA versus transcriptional profiling. However, each method of pathway analysis has its place. MPA may serve a good purpose in illuminating fine-tuned regulation in the AF/ST pathway itself, and lead to insights regarding why different cultures produce different amounts of mycotoxin even though they are grown under similar supporting conditions.

CONCLUSION

Fungi are amazing organisms, and can produce a wide array of secondary metabolites. Some are helpful, such as the drugs used to control cholesterol levels in humans, and antibiotics. Still, many secondary metabolites produced by fungi are toxic to plants and/or animals, and the understanding of how they are produced and regulated will benefit society greatly.

Much has been learned in the past 40 years of studying aflatoxin production by Aspergillus species, yet there is still much to be discovered about the regulation of this
impressive toxin. New tools such as metabolic pathway analysis and transcriptional profiling hold promise for accelerating our understanding of aflatoxin production, and providing potential targets for control. Hopefully these new technologies may even allow us to change these pathways in fungi, and produce novel compounds which will benefit mankind for years to come.
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Aflatoxin conducive and non-conducive growth conditions reveal new gene associations with aflatoxin production.*

Michael S. Price¹, Shannon B. Conners², Sabrina Tachdjian², Robert M. Kelly² and Gary A. Payne¹*.

¹Center for Integrated Fungal Research, North Carolina State University, Raleigh, NC 27695-7244.
²Department of Chemical Engineering, North Carolina State University, Raleigh, NC 27695-7905.

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Abstract

Research on aflatoxin (AF) production has traditionally focused on defining the AF biosynthetic pathway with the goal of identifying potential targets for intervention. To understand the effect of nitrogen source, carbon source, temperature, and pH on the regulation of AF biosynthesis, a targeted cDNA microarray consisting of genes associated with AF production over time was employed. Expression profiles for genes involved in AF biosynthesis grouped into five clades. A putative regulon was identified consisting of 20 genes that were induced in the conducive nitrogen and pH treatments and the non-conducive carbon and temperature treatments, as well as four other putative regulons corresponding to each of the four variables studied. Seventeen genes exhibited consistent induction/repression profiles across all the experiments. One of these genes was consistently downregulated with AF production. Overexpression of this gene resulted in repression of AF biosynthesis. The cellular function of this gene is currently unresolved.

Keywords: aflatoxin, gene expression, regulation, Aspergillus flavus, Aspergillus parasiticus
Introduction

Aflatoxins (AF) are carcinogenic polyketides produced during secondary metabolism by five species of Aspergilli: *A. flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, and *A. bombycis* (Samson, 2001; Varga et al., 2003). Only two of these species, *A. flavus* and *A. parasiticus*, are of agronomic importance and cause annual losses of approximately $270M (Richard and Payne, 2003). These fungi cause pre-harvest rots on corn, peanuts, various tree nuts, and cotton. Furthermore, they are a post-harvest problem in many areas of the world. The main concern with these fungi is not the disease they cause, but their ability to contaminate food with AF.

The regulation of AF biosynthesis has been studied since 1965, when a possible role of kojic acid in AF production was investigated (Heathcot et al., 1965). Since that time, the biosynthetic pathway for AF has been elucidated and a specific transcriptional regulator has been characterized (Chang et al., 1993; Woloshuk et al., 1994). Regulation appears to involve several gene products and is linked to fungal development (Calvo et al., 2002). In addition, many nutritional and environmental factors are known to influence AF production, including nitrogen source (Kachholz and Demain, 1983), carbon source (Davis and Diener, 1968; Buchanan and Stahl, 1984), culture temperature (Schindle et al., 1966; Diener and Davis, 1967; Hunter and Tuite, 1967; Mayne et al., 1967; Schindle et al., 1967), and culture pH (Cotty, 1988; Keller et al., 1997). Organic sources of nitrogen and simple sugars favor AF production, while inorganic nitrogen sources and complex carbohydrates retard biosynthesis. The growth-optimal temperature of 37°C inhibits AF biosynthesis completely, while 28°C allows production. Conversely, the growth-optimal pH 4.5 is conducive for AF biosynthesis and production is inhibited at pH 8.0. Although the generalities are known, the specific regulatory circuits that enable these factors to control AF production remain unknown.

cDNA microarray analysis has rapidly become an invaluable tool for defining regulatory networks in cells (Alizadeh et al., 1999; Alizadeh et al., 2000; Welch et al., 2002). Microarrays also are used to identify genes involved in cellular processes, such as pathogen defense (Cohen et al., 2000; Moran et al., 2002; Ramonell et al., 2002; de Torres et al., 2003; Golem and Culver, 2003; Whitham et al., 2003), infection (Takano et al., 2003),
and growth (DeRisi et al., 1997; Lashkari et al., 1997; Pennie, 2000). Perhaps the greatest power of microarray technology lies in two-way clustering of genes and treatments to identify genes with common transcriptional profiles. Hughes et al. (2000) demonstrated this power by clustering expression data from 300 different array experiments using the yeast *Saccharomyces cerevisiae* and identifying previously unknown ergosterol biosynthesis genes.

In this study, we utilized a targeted cDNA array of clones previously demonstrated to be correlated with AF biosynthesis (OBrian et al., 2003) to investigate the influence of nitrogen source, carbon source, culture temperature, and culture pH on AF production and gene transcription. The differential expression data for each experiment were clustered to produce a small compendium of expression profiles, which identified genes with expression profiles similar to the AF cluster genes. Furthermore, new gene associations were made to AF production via the identification of consistently induced or repressed genes with respect to AF biosynthesis. The genes identified in this manner have not been previously associated with AF production.

**Materials and Methods**

**Fungal strains and culture conditions.**

Conidia (10^6/mL) of *Aspergillus parasiticus* (Speare) strain SU1 (ATCC 56775) were inoculated into 2 L flasks consisting of either 500 mL YES broth (2% yeast extract, 5% sucrose, pH 4.5) or YEL broth (2% yeast extract, 5% lactose, pH 4.5) plus 0.4% agar. The addition of agar maintained confluent growth in submerged culture. Seed cultures were grown in shake culture (200 rpm) for 16 hours at 28°C in all experiments except the temperature study, where the seed cultures were grown at 37°C. A 6.25 mL aliquot of the seed cultures was used to inoculate daughter cultures (100 mL each) in the nitrogen, temperature and pH studies using SLS broth (85 g/L sucrose, 10 g/L asparagine, 50 mL/L 20x Salts [70 g/L (NH₄)₂SO₄, 15 g/L KH₂PO₄, 7 g/L MgSO₄·7H₂O, 1.5 g/L CaCl₂·2H₂O, 0.4 g/L ZnSO₄·7H₂O, 0.1 g/L MnCl₂·4H₂O, 0.04 g/L (NH₄)₆Mo₇O₂₄·4H₂O, 0.04g/L Na₂B₄O₇, 0.04 g/L FeSO₄·7H₂O) as the base medium. YEL seed cultures were used to inoculate daughter cultures (100 mL each) in the carbon study.
Conditions conducive for AF production examined in this study were as follows: NH₄ as the nitrogen source, sucrose as the carbon source, a culture pH of 4.5, and a culture temperature of 28°C. Therefore, SLS medium was used as the conducive medium, with constituents altered individually for each non-conducive medium. For example, in the nitrogen source comparison, the media were identical for pH (pH 4.5), carbon source (sucrose), and temperature (28°C), with the only difference being in nitrogen source (ammonium or nitrate). For the nitrogen source cultures, nitrogen was included in the daughter culture medium as either ammonium or nitrate at 8.24 g/L nitrogen (using (NH₄)₂SO₄ or NaNO₃, respectively). This is similar to the amount of nitrogen used in a previous study that examined the repression of AF and averufin biosynthesis by nitrate (Kachholz and Demain, 1983). For the carbon source cultures, either sucrose or lactose was included at 46.5 g/L carbon. For the pH experiment, SLS medium was prepared and buffered at either pH 4.5 (using 100 mM citric acid) or pH 8.0 (using 100 mM Tris base). The temperature experiment utilized SLS medium, with the cultures incubated at either 28°C or 37°C.

The pH of the nitrogen, carbon, and temperature daughter cultures was maintained at pH 4.5 by buffering with 100mM citric acid. The nitrogen, carbon, and pH daughter cultures were incubated at 28°C. All daughter cultures were incubated for 24 hours and harvested for RNA preparation and AF quantification. AF concentrations for each culture were determined by HPLC. Triplicate cultures were grown for each treatment.

**RNA preparation.**

Tissue was isolated from cultures by vacuum filtration. Tissue from individual culture replicates were placed into pre-weighed 50 mL conical tubes and stored at -80°C prior to lyophilization. Tissue samples were lyophilized for 24-48 hours to remove water, weighed, and stored at -80°C until use.

For RNA extraction, replicate tissue samples for a given treatment were pooled and ground with liquid nitrogen in a mortar with a pestle. RNA was then extracted from the pulverized tissue using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA).
following manufacturer’s instructions. RNA preparations were then visualized by gel electrophoresis and quantified by spectrophotometry.

**Microarray construction and target labeling.**

The microarrays used in this study were constructed as described previously (OBrian et al., 2003), with the following exceptions. The PCR products were resuspended in 50% DMSO and printed in sextuplet onto Corning UltraGAPS slides using a Genetix Qarray Mini array printer (Genetix Limited, Hampshire, UK). A pool of clones from the cDNA library used for printing was printed as a positive control. In addition, the AF pathway genes aflJ and aflR, as well as genes laeA (from A. nidulans), pkaA, pacC, areA, creA, and brlA were printed as positive controls. The gene crip3 (from maize) was printed as a negative control. Therefore, a total of 763 genes were represented on the slides. Printed slides were dried overnight before UV cross-linking per manufacturer’s instructions.

Total RNA from *A. parasiticus* SU1 was converted to first strand cDNA using Stratascript™ reverse transcriptase (Stratagene, La Jolla, CA) and random hexamer primers (Invitrogen Life Technologies, Carlsbad, CA) with the incorporation of aminoallyl dUTP (Ambion Inc., Austin, TX) as described elsewhere (Chhabra et al., 2003) for all experiments except the first nitrogen source experiment. This array was labeled with the different Cy-dyes using the Genisphere Array 350 kit (Genisphere Inc., Hatfield, PA) and following manufacturer’s instructions. Each cDNA sample for each treatment was labeled once with each dye to account for differences in dye labeling.

**Microarray hybridization, data acquisition, and analysis.**

Cy-dye-labeled cDNAs were hybridized to the microarrays for 16-20 hours at 42°C for each growth variable set. The hybridized slides were scanned using a Perkin Elmer ScanArray Express Lite scanner (Perkin Elmer Life and Analytical Sciences Inc., Boston, MA). Pixel intensity data were extracted from the resulting image files using Scanalyze, a freeware microarray analysis program (http://rana.lbl.gov/EisenSoftware.htm). The microarray data produced in this study was deposited in the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) under accession numbers GSM27953, GSM27954, GSM27956, GSM27958, GSM27959, GSM27961,
GSM27963, GSM27964, GSM27967, GSM27969, GSM27971, GSM27973, GSM27975, GSM27977, GSM27978, and GSM27979.

After extraction, data were imported into a statistical software package (SAS v8, SAS Institute, Cary, NC), and initial background subtraction was performed. Mixed model analysis of microarray data (Wolfinger et al., 2001) was used to evaluate differential expression data using approaches presented elsewhere (Chhabra et al., 2003; Pysz et al., 2004). Briefly, the mixed procedure in SAS was used to implement a linear normalization ANOVA model to estimate global variation in the form of fixed (dye (D), treatment (T)) and random (array (A), block (A(B)), spot (A(S x B))) effects and random error using the model

$$\log_2(y_{ijklmn}) = \mu + A_i + D_j + T_k + A_i(B_l) + A_i(S_m x B_l) + \varepsilon_{ijklmn}.$$  

A gene-specific ANOVA model was then used to partition the remaining variation into gene-specific effects using the model

$$r_{ijklm} = \mu + A_i + D_j + T_k + A_i(B_l) + A_i(S_m x B_l) + \varepsilon_{ijklm}.$$  

Least squares estimates of gene-specific treatment effects across pairs of treatments were obtained for each gene under each treatment condition. Differences between treatment effects for pairs of conducive and non-conducive conditions can be considered as log2-transformed fold changes (Wolfinger et al., 2001). Hierarchical clustering of selected log2-transformed fold changes from two biological repetitions of all conditions of interest was performed using Ward’s minimum variance method in JMP 5.0 (SAS Institute Cary, NC). Genes were selected for hierarchical clustering if they possessed fold changes greater than 1.7 or less than -1.7 in at least one experiment. This resulted in the inclusion of 542 genes in the clustering. Genes were designated as significantly different in the individual microarray experiments if they possessed p-values less than \(\alpha\). The value of \(\alpha\) (0.003) was chosen based on the significance of the AF pathway genes included on the array.

**Overexpression of CA747470, a gene involved in AF production.**

The Universal GenomeWalker™ kit (BD Biosciences Clontech, Palo Alto, CA) was used to determine the genomic sequence flanking EST CA747470 (Figure 5). This genomic sequence data was analyzed by the FGenesH gene prediction software package* using “Aspergillus” as the organism. Primers were designed 100bp upstream and downstream

* http://www.softberry.com
from the predicted coding region, and were used to clone the putative gene into pNOM102, an expression vector containing the gpdA promoter and A. nidulans trpC terminator sequences. The resulting construct was designated p01H11OE.

p01H11OE was co-transformed into A. flavus 3357-5 (pyrG) with pBS-pyr4, a construct containing the N. crassa pyr4 gene using a PEG-based protocol previously described (Woloshuk et al., 1989). Seventeen transformants restored to uracil prototrophy were picked onto PDA (Potato Dextrose Agar; Becton Dickinson & Co., Sparks, MD). The transformants were screened for the presence of the p01H11OE construct using PCR primers designed to amplify a region from the pNOM102 backbone into the CA747470 insert. Four of the seventeen transformants were identified as PCR-positive for having the overexpression construct (transformants 1-3, 3-1, 3-2, and 3-3).

The PCR-positive transformants were tested for AF production. Agar plugs (3mm diameter) taken from growing cultures of the transformant strains 1-3, 3-1, 3-2, and 3-3, as well as 3357-5 and WT strain 3357. Eight plugs each were inoculated into twin 50 mL potato dextrose broth (PDB) cultures (one set per strain). These cultures were grown at 28°C, 200 rpm for 48 hrs. Culture filtrates were tested for AF as described above.

Quantitative-PCR (qPCR) data were collected for transformant strains 1-3, 3-2, and 3-3, as well as 3357-5. The primers 5’-AAACCCACAAGGCAACAT-3’ and 5’-CTGGTTCTTGACCCAAAGGT-3’ were used to amplify an 88 bp region of the putative coding sequence of CA747470. The program used for PCR was as follows: 1 cycle of 95°C x 10 min; 40 cycles of 95°C x 15 sec, 53.8°C x 30 sec, 72°C x 1 min. Raw Ct values for CA747470 expression were by normalized subtraction of 18S control values by strain, then standardized by subtraction of the wild type Ct value from the transformant Ct values, yielding a log₂ expression value. The fold-change is obtained by raising 2 to the power represented by the log₂ expression value for a given strain.

Lastly, in order to test the effect of overexpression of CA747470 on growth, the four PCR-positive transformants along with A. flavus wild type strains 3357-5 and 3357 were picked onto minimal media plates (MM; Czapek-Dox medium, Becton Dickinson & Co, Sparks, MD) amended with each of the following: 0.5M KCl, 1.0M KCl, 0.5mM CuSO⁴, or
5mM CuSO4. In addition MM was prepared with final pH as pH 3.0 or pH 8.0. The plates were incubated at 28°C for 5 days. Colony diameter was measured as the mean of cross-wise measurements for each of three replicate plates for each strain on each medium. Measurements were taken approximately the same time every day for 5 days. The results of growth on day 5 are reported.

Figure 1. Aflatoxin production by *A. parasiticus* grown in conducive or non-conducive culture treatments. Data presented are for two biological replicates for each culture condition. Three replicate cultures for each biological replicate were grown for 24 hrs. and harvested for aflatoxin quantification and RNA extraction. Values presented are the means of the six replicate cultures. Error bars represent standard error for each treatment.
Results and Discussion

Aflatoxin production in growth cultures.

We were able to assay for AF production in each culture, and use this as a phenotypic screen for the microarray experiments. We consistently observed large statistically significant differences in AF production between conducive and non-conducive treatments for each experiment (Figure 1). In fact, no AF was made at 37°C or at pH 8.0. While some AF was observed in cultures grown under non-conducive carbon and nitrogen conditions, the level of AF repression by nitrate was similar to that reported previously (Kachholz and Demain, 1983). The level of AF in the lactose-containing cultures was over 8-fold higher than previously reported by Buchanan and Stahl (Buchanan and Stahl, 1984), however they included 75% less lactose in their culture medium. These differences in carbon source amounts may have had an impact on AF production beyond the impact of the specific carbon source they used.

Agreement of microarray data with previously published results.

The array data from this study were compared to results from a previous study that examined the influence of fungal development on AF production (OBrian et al., 2003). OBrian and colleagues examined the change in AF gene expression over time by comparing transcripts from tissue grown for either 8 hrs (no toxin) or 24 hrs (toxin). These time-points correspond to primary and secondary growth, respectively. Of the 42 differentially expressed genes identified in the OBrian study, 37 genes were differentially expressed in the microarrays presented here (Figure 2). This group of 37 genes was subjected to clustering as described in Methods.

As shown in Figure 2A, many of these genes had unique expression patterns. However, one group of genes was identified with nearly identical expression profiles, and three of the genes in this group exhibited differences that met our significance criteria in all four culture variable comparisons (purple box, Figure 2A). Preliminary analysis of the upstream sequences of these genes revealed the presence of creA, areA, and/or5 pacC transcription factor binding sites. This group of genes is part of a larger cluster in this study consisting of 20 genes (Figure 2B), most of which have not been previously described (NSH,
Figure 2. Behavior of genes in this study previously shown to be differentially expressed over time with regard to AF production. (A) A subset of 42 genes shown to be differentially expressed in a prior temporal expression experiment (OBrian et al., 2003) was identified and subjected to hierarchical clustering as described in Methods. Differences in gene expression from the earlier study are qualitative and represented as red (higher in conducive treatment) or blue (higher in non-conducive treatment). The highlighted clade (purple box) represents a possible regulon. (B) The genes highlighted in (A) were identified in the original clustering (used for Figure 5), and reside in a larger group of genes with a similar expression profile, suggesting a putative regulon of 20 genes.
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**Diagram**

- **A**: Accession Clustering Putative Protein ID, OBrian, 2003
- **B**: nitrogen, pH, temperature, carbon, log2R

- **3.6** - **0** - **-3.5**
No Significant Homology). The high degree of similarity in transcription profiles suggests these genes constitute a putative regulon. These genes may share transcription factor binding sites leading to their coordinate regulation, or there may be a cascade effect among genes in this cluster, whereby certain genes affect the expression of other genes within the cluster to the same extent by which they are expressed. Alternatively, if the protein products of these genes are part of the same metabolic pathway, changes in product flux through that pathway may yield like effects on the transcription of all these genes through a mechanism similar to feedback inhibition.

This regulon has the unique characteristic of being upregulated in the conducive nitrogen source and pH, and suggest these genes are involved in cross-talk between nitrogen and pH regulatory pathways. A link between nitrogen repression and pH has been examined (Cotty, 1988). Cotty showed that four-fold less AF was produced in cultures containing ammonium which were buffered at pH 5.5 than ammonium-containing cultures which were not buffered. While this cross-talk may be due solely to the apparent tendency of ammonium containing cultures to produce lower pH, the fact that a set of genes are upregulated in response to both environmental cues suggests that some amount of pathway cross-talk may in fact exist.

**Identification of treatment-specific regulons.**

Suites of genes were identified which corresponded to specific culture variables tested in this study (Figure 3). Carbon regulation of AF production has been studied since 1968, when the effect of 13 carbon sources on AF accumulation by cultures of *A. parasiticus* was investigated (Davis and Diener, 1968). The authors suggested that for a carbon source to support both growth and AF production, it must be accessible to both the hexose-monophosphate and glycolytic pathways. We identified a regulon consisting of genes upregulated in response to sucrose and associated with AF production (Figure 3A). Not surprisingly, one of the genes (*enoA*) in this cluster has homology to enolase, which is responsible for the conversion of glycerate-2-P to phosphoenol-pyruvate in the glycolytic pathway. Another gene (*pdcA*), putatively a pyruvate decarboxylase, is involved in the
Figure 3. Identification of putative culture-condition responsive regulons. (A) A subset of genes that exhibited higher expression in response to sucrose. Included in this clade are a putative enolase as well as a putative pyruvate decarboxylase. The genes shown exhibited between 2.3 and 3.9-fold induction in response to growth on sucrose. (B) A group of 27 genes, including the global pH regulatory gene pacC, that exhibited higher expression at pH 8.0. (C) A set of 18 genes, including a putative coproporphyrinogen III oxidase, that exhibited higher expression in response to nitrate. (D) A set of three genes of unknown function, as well as three putative superoxide dismutases, that exhibited increased expression due to growth at 37°C. Genes with no putative function are designated NSH.
related gluconeogenesis pathway. The five unique (NSH) genes in this cluster provide tempting targets for further study of carbon regulation.

Much work has been done investigating the role of pH and regulation of AF biosynthesis (Keller et al., 1997; Ehrlich et al., 1999). The binding site for the global pH regulator PacC has been identified in the promoter of aflR (Ehrlich et al., 1999). Our study identified 27 genes coordinately induced with increasing pH (Figure 3B). Included in this group is the global regulatory gene pacC. This gene is known to induce expression of alkaline-expressed genes while repressing expression of acidic-expressed genes. Putative PacC binding sites were observed in the upstream sequences of 19 genes in this cluster (data not shown), further strengthening the argument these genes constitute a putative regulon. This gene cluster also included pkaA, which has been shown to inhibit AF production via phosphorylation of AFLR (Shimizu et al., 2003). pnc1, a gene involved in nicotinamide metabolism, was also induced by high pH. This finding is intuitive because this pathway is involved in the production of NAD$^+$ and NADP$^+$, both of which accept hydride ions in enzymatic reactions, resulting in the release of protons. This may be one way in which the fungus balances intracellular pH in an alkaline environment.

Nitrate repression has likewise received much attention with respect to AF production (Kachholz and Demain, 1983; Niehaus and Jiang, 1989; Chang et al., 1995; Flaherty and Payne, 1997; Feng and Leonard, 1998; Liu and Chu, 1998; Ehrlich and Cotty, 2002). As early as 1983, Kachholz and Demain showed that nitrate, in a pH buffered medium, was capable of suppressing averufin production up to 70% compared to ammonium. Niehaus and Jiang (1989) later showed that the addition of nitrate to a growing culture caused a concomitant increase in activity of mannitol dehydrogenase and mannitol-1-P dehydrogenase (2.9 and 7.7-fold, respectively). These enzymes are capable of making NADPH and NADH, respectively, but are most likely not major sources of these cofactors in the cell. The authors argue that nitrate repression occurs due to an altered redox state in the cell, as measured by NADPH/NADP$^+$ ratio.

No genes have been identified to date that are responsible for regulating nitrate repression of AF biosynthesis. In this study we identified a potential regulon of genes which
are coordinately induced by nitrate (Figure 3C). One gene, \textit{cpx}, has homologs which are involved in heme production. Heme is a structural component of nitrate reductase, which is the first enzyme in the nitrogen assimilation pathway (Marzluf, 1997). Upregulation of this gene is intuitive, as nitrate reductase is required for nitrate utilization. Three other genes, \textit{cox12}, \textit{hmpA}, and \textit{fhp}, encode proteins involved in the electron transport chain, which is responsible for production of energy and reducing power in the cell. Reducing power is essential for nitrate utilization, therefore increased expression of these genes is expected. Again, similar to the putative pH regulon, 16/18 genes identified in this cluster possess putative binding sites for the global nitrogen regulatory protein AreA (data not shown). This evidence bolsters our confidence in these genes being co-regulated.

Temperature regulation of AF production has been studied since the late 1960’s, when Schindle and colleagues first described the conducive temperatures for AF (Schindle et al., 1966). The effect of high temperature on \textit{aflR} transcription has been well established (Feng and Leonard, 1998; Liu and Chu, 1998), yet a degree of post-transcriptional regulation cannot be ruled out. In the present study a small putative regulon consisting of six genes was identified that responded to growth at 37°C. Three of the genes encode putative superoxide dismutase genes (MnSOD and \textit{sodM}; Figure 3D). This finding may reflect the stress encountered by the fungus during growth at higher temperatures, or its need to escape host defenses during plant or animal infection. The other genes in this cluster are undefined (NSH), yet investigations into their function may yield interesting insights into temperature regulation of AF biosynthesis.

\textit{Expression profiles of AF pathway genes.}

Transcriptional profiles of the AF genes on this microarray clustered into five clades (Figure 4). The structural gene clade contained genes responsible for some of the enzymatic steps of the AF pathway, including \textit{aflC} (formerly \textit{pksA}; 2 copies), \textit{aflJ} (formerly \textit{estA}), \textit{aflM} (formerly \textit{ver1}; 2 copies), \textit{aflP} (formerly \textit{omtA}), and \textit{aflO} (formerly \textit{omtB}). Also included in this clade was one gene encoding a putative 6-phosphogluconate dehydrogenase. These genes exhibited large increases in expression when grown under the conducive conditions tested.
Figure 4. Hierarchical clustering of AF pathway genes. Differential expression data for genes meeting cutoff criteria (difference estimate >0.75 or <0.75) were grouped using Ward’s hierarchical clustering as described in Methods. Representative AF pathway genes exhibited different expression profiles and were grouped into five clades.
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**Nitrogen, Temperature, pH, Carbon**

![Heatmap with log2R scale]
Another clade with similar but less striking differences in gene expression is the aflS clade. aflS (formerly aflJ) is divergently transcribed from the pathway specific regulator aflR (Meyers et al., 1998). This gene was found in a separate cluster from the other AF pathway genes with one gene of unknown function (NSH), two putative actin subunits, a ribosomal subunit, and a putative transaldolase (Figure 4). While the differences in aflS gene expression between treatments were not as great as the main structural genes, the observed differences correlated with AF production.

A recently described pathway gene, aflH (formerly adhA) shared a similar transcription profile with four genes homologous to ribosomal proteins and one gene homologous to cystathionine β-synthase. aflH has been recently demonstrated to convert 5’-hydroxyaverantin (HAVN) to averufin (AVF) via a 5’-oxoaverantin (OAVN) intermediate in the AF biosynthetic pathway (Chang et al., 2000; Sakuno et al., 2003). Specifically, AflH was shown to convert HAVN to OAVN. The conversion of OAVN to AVF was shown to be

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*Gene did not meet criteria (p-value<0.003, log₂R>0.75 (induced) or log₂R<-0.75 (repressed)) for one replicate of one experiment.
the result of the action of OAVN cyclase, a protein not encoded by any AF pathway gene (Sakuno et al., 2003). Cystathionine β-synthase is an enzyme involved in cysteine synthesis and sulfur metabolism. This enzyme is responsible for producing L-cysteine and acetate in the sulfur metabolic pathway. Acetate is believed to be a precursor to AF production in A. flavus (Brown et al., 1999).

The early pathway gene aflD (formerly norI) had a unique expression profile, and shared an expression profile with five other genes including one gene of unknown function (NSH), a gal10 homolog, a ribosomal protein homolog, and two genes with homology to A. fumagatus allergen rasp f13. The pathway specific transcriptional regulator aflR exhibited an expression profile unlike other pathway genes, and was grouped in a clade with three NSH genes, a ribosomal protein gene, and a gene with homology to snaD, which is involved in asexual sporulation in A. nidulans (Liu and Morris, 2000).

In addition to finding genes with similar expression profiles to known AF biosynthetic genes, other genes were identified which were consistently associated with AF production in all the experiments performed (Table 1). Three of the genes on our array were consistently repressed with respect to AF production. Two of these genes were uncharacterized (i.e. NSH), while one had homology to a nitrogen-starvation induced cDNA from Colletotrichum gloeosporioides. Conversely, fourteen genes were induced with respect to AF production. These included the AF pathway genes aflC, aflM, and aflP, as well as genes involved in other cellular processes and three NSH genes.

Least squared mean estimates of treatment effects representing transcript detection levels in each of the treatments tested were plotted by clone for genes of interest identified in Table 1, as well as two other genes with interesting putative functions (Figure 5). Three of the AF biosynthetic genes are shown: CA747495 (aflC), CA747496 (aflM), and CA747926 (aflP). Two other genes, CA747645 and CA747794 (encoding putative transaldolase (talI) and 6-phosphogluconate dehydrogenase (pgd), respectively), were also identified in Table 1 and are of interest due to their possible role in the oxidative stress response and nitrate utilization pathways. Oxidative stress has been hypothesized as necessary for AF production (Jayashree and Subramanyam, 2000). Further, pgd is thought to be a major source of
Figure 5. Relative expression levels in all treatments for select genes of interest. Least squared means estimates of expression obtained from the mixed-model analysis of the microarray data (see Methods for explanation and for relationship to fold changes) were plotted for select genes of interest. Included in the plot are three AF pathway genes (CA747495, CA747496, and CA747926) as references.

NADPH in the cell, and NADPH availability is hypothesized to be important for AF production (Niehaus and Jiang, 1989). Disruption of these genes is underway in the hopes of determining their role in AF biosynthesis.

Many fungi that produce mycotoxins possess mechanisms for mitigating self-toxicity. Cercospora kikuchii has a cercosporin pump which is involved in self-defense against this mycotoxin (Callahan et al., 1999; Upchurch et al., 2001), and a gene for singlet oxygen resistance has been shown to confer cercosporin resistance in C. nicotianae (Ehrenshaft et al., 1997; Ehrenshaft et al., 1999). A recently identified MFS transporter located in the AF biosynthetic cluster, aflT, was shown to play no role in AF secretion or resistance (Chang et al., 2004). We identified a gene in our microarray clone set with homology to aflatoxin
aldehyde reductase (AFAR; CA748087), which has been shown to be responsible for AF resistance in a rat model (Hayes et al., 1993). This gene exhibited high expression in all treatments except 37°C (Figure 5). The presence and expression of this putative AFAR gene in all treatments suggests a role in AF resistance by *A. flavus*. Gene-deletion experiments are underway to verify a role for CA748087 in AF resistance.

Two genes in Figure 5 were shown in Table 1 to be consistently downregulated with respect to AF: CA747470 and CA747569. One of these genes showed no significant homology to any known genes, proteins, or protein domains (CA747470), whereas the other gene exhibited homology to a nitrogen starvation induced cDNA from *Colletotrichum gloeosporioides* (CA747569). Induction of CA747569 in nitrate medium were also observed here, however there appears to be a greater transcriptional response between conducive and non-conducive temperature and carbon source treatments. In the case of CA747470, transcription of this gene was much higher than the others in Figure 5 in each treatment, suggesting that perturbations in expression of this gene may well yield a visible phenotype.

**Identification of CA747470, a gene involved in AF production.**

We identified a gene, CA747470, whose expression was inversely correlated to AF production. As noted above, this gene has no homology to known sequences and is highly expressed in all conditions presented here (Figure 5). We designed an overexpression construct, p01H11OE, to investigate the effect of elevated expression of this gene on AF production under conducive conditions. Four transformants were obtained which possessed the construct and were restored to uracil prototrophy. Of these, one transformant exhibited a

<table>
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*Change in expression level for CA747470 with respect to 3357-5
“fluffy” growth phenotype similar to the previously described flbA developmental mutant (Yu et al., 1996). Due to this similarity to previously established developmental mutations, 3-1 was not investigated further.

As shown in table 2, CA747470 overexpression was detected by qPCR. Interestingly, strain 1-3 produced near wild-type levels of AF, while strains 3-2 and 3-3 produced 8% and 0.3% of wild-type AF levels, respectively. This gene appears to be highly expressed natively, even under conducive AF conditions. Therefore activity of the CA747470 gene product may be controlled by a mechanism like protein phosphorylation. If gene expression outpaces inactivation sufficiently, then AF production is reduced. This may explain how 1-3 makes near wild-type levels of AF while CA747470 is overexpressed 67-fold. In strains 3-2 and 3-3, this gene is overexpressed more than 90-fold, and conceivably may outpace the cells ability to suppress its activity. Furthermore, The amount of fold change appears to inversely correlate to reduction in toxin (r² = 0.8). These data taken as a whole suggest that CA747470 is involved in either suppressing or delaying AF production.

Finally, the effect of overexpressing CA747470 on growth was determined using MM amended with CuSO₄ or KCl, or by changing pH. These amendments were chosen to simulate oxidative stress, salt-stress, and AF conducive and non-conducive pH, respectively. Transformant strains 3-2 and 3-3 grew better than 3357-5 in every case except on 5mM CuSO₄ (Figure 6). In all cases, 3-2 and 3-3 grew as well as or better than the parental wild-type strain 3357. These data suggest that CA747470 may play a role in vegetative growth. Overexpression of this gene may mimic vegetative growth signals in the fungus, which results in suppressed AF production. Further studies of this gene are underway.

In conclusion, we have used a targeted cDNA microarray to examine transcriptional differences in A. parasiticus due to conducive and non-conducive conditions for AF production. Differences in gene expression between AF pathway genes within treatments were revealed, with the AF pathway genes exhibiting 5 different patterns of gene expression. Genes were identified with patterns of expression similar to the AF pathway genes (Figure 4). These newly identified genes are of interest for further investigation. Genes were also identified which respond to the treatments evaluated in this study (Figure 3), and will be
Figure 6. Growth of CA747470 overexpression transformants on MM supplemented with various amendments. Radial growth was recorded as the mean of crosswise measurements from three replicate plates after 5 days incubation at 28°C. Strains are designated as follows: transformant strain 1-3, 3-2, 3-3; wild type strains 3357, 3357-5.

targets for investigating the role of carbon source, etc. in secondary metabolism. Furthermore, we identified a gene, CA747470, whose expression was inversely correlated to AF production (Figure 5, Table 2). Overexpression of this gene resulted in lower AF production, suggesting this gene may play a role in regulating the AF pathway. CA747470 appears to be involved in vegetative growth, which may explain its negative effect on AF production. Future experiments on this gene will involve gene deletion and microarray studies to determine the effect of CA747470 overexpression and deletion on the transcriptome.
AF production by species of *Aspergillus* is a highly regulated process, involving multiple layers of transcriptional and post-transcriptional regulation (Buchanan et al., 1987; Woloshuk et al., 1994; Hicks et al., 1997; Keller et al., 1997; Feng and Leonard, 1998; Butchko et al., 1999; Chang et al., 1999; Shimizu and Keller, 2001; Calvo et al., 2002; Chiou et al., 2002; Shimizu et al., 2003). Examples of regulatory cross-talk between growth conditions are also observed (Cotty, 1988). All of this regulation is focused through the AF pathway-specific transcriptional regulator *aflR* (Chang et al., 1993; Payne et al., 1993). In the last ten years much progress has been made in understanding the regulation and production of AF in *A. flavus* and sterigmatocystin (ST) in *A. nidulans*. Virtually all of the enzymatic steps have been characterized (Sakuno et al., 2003; Price and Payne, 2004). However, much remains to be described about the regulatory networks involved in AF biosynthesis, and how other cellular processes are influenced by these networks. This study has just begun to uncover the regulatory network influencing AF production and growth in *Aspergilli*. Future work will include time-dependent analysis of AF-conducive conditions utilizing arrays with larger numbers of genes. The research presented here should lay a firm foundation upon which further exploration of the factors influencing AF biosynthesis may be conducted.

**Acknowledgements**

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References


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The aflatoxin pathway regulatory gene \textit{aflR} regulates genes outside of the aflatoxin biosynthetic cluster.\textsuperscript{*}

Michael S. Price\textsuperscript{1}, Jiujiang Yu\textsuperscript{2}, Deepak Bhatnagar\textsuperscript{2}, Thomas E. Cleveland\textsuperscript{2}, William C. Nierman\textsuperscript{3}, Bethan Pritchard\textsuperscript{1}, and Gary A. Payne\textsuperscript{1}

\textsuperscript{1}North Carolina State University, Center for Integrated Fungal Research, Box 7567, Raleigh, NC 27695-7567.
\textsuperscript{2}Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, New Orleans, LA 70124.
\textsuperscript{3}The Institute for Genomic Research, Rockville, MD 20850.

\textsuperscript{*} To be submitted to Applied and Environmental Microbiology.
Abstract

The carcinogen aflatoxin is synthesized by a cluster of genes that are regulated by the transcriptional factor, AflR. Most, but not all of these genes, have a consensus binding site for AflR in their 5’ untranslated region. Because aflR resides within the biosynthetic cluster, it has been suggested that it regulates only genes within the cluster. The objective of this study was to identify those genes transcriptionally regulated by AflR and to determine if any of those genes reside outside the aflatoxin biosynthetic cluster. To address this objective, we employed a cDNA microarray of 5,002 genes from Aspergillus flavus to monitor the expression of genes in a wild type and an aflR deletion strain of A. parasiticus. Nineteen of the twenty-five known genes in the cluster were more highly expressed in the wild type strain. These included all of the genes reported to have a consensus binding site for AflR, as well as other genes within the cluster. This research also identified a new gene within the cluster that has an AflR consensus binding site and is regulated by AflR. Further, nadA, a gene previously reported to be in an adjacent sugar cluster, was found to contain the consensus AflR binding site and to be upregulated in the wild type strain. Finally, two genes that were regulated by AflR in this study lie distant to the aflatoxin cluster. These two genes, one that encodes nitrite reductase and the other that is predicted to encode a hemolysin, were also found to possess an AflR binding site.

Introduction

Since the 1960’s, aflatoxin biosynthesis has been studied in an effort to reduce contamination of crops with this mycotoxin. Pre- and post-harvest aflatoxin contamination of crops is a chronic problem in many developing countries of the world, and remains a concern in industrialized nations. Much was learned about the production of aflatoxin throughout the 1960’s and 1970’s [1-6], yet our understanding of the regulation of aflatoxin production was limited. The discovery of the pathway specific regulator, AflR, in the 1990’s opened the door for investigations into the genetic regulation of the aflatoxin pathway. Much progress in understanding this regulation has been made with respect to development [7], and the role of nutrient and environmental cues [8-11]. AflR has been demonstrated to regulate transcription of many aflatoxin pathway genes [12, 13]. In addition, AflR is post-
transcriptionally regulated via PkaA [14], transcriptionally regulated [14], and is believed to autoregulate itself [15]. It is unknown whether the pathway specific transcriptional regulator AflR is able to regulate the expression of genes outside the aflatoxin biosynthetic pathway.

With the advent of cDNA microarrays technologies in the late 1990’s, we are now able to conveniently investigate the effect of treatments on the entire transcriptome of cells, or any subset thereof [16-22]. cDNA microarrays are a powerful tool, allowing us to identify new genes involved in cellular processes such as ergosterol biosynthesis [23] by examining profiles of gene expression across treatments. This method of examining gene expression in A. parasiticus has lead to the identification of targets associated with aflatoxin biosynthesis in need of further investigation [11].

In this study we examined the effect of deletion of aflR on the transcriptome of A. parasiticus using a cDNA microarray representing approximately 40% of the estimated A. flavus gene complement. Expression data for this experiment were collected at three different timepoints, and clustered with previous data [11] to produce a small compendium of gene expression data. New genes responding to aflR deletion were identified which reside apart from the established aflatoxin biosynthetic cluster. Furthermore, we suggest that the aflatoxin biosynthetic cluster be expanded to include additional genes that reside at the biosynthetic pathway locus and are under the control of AflR. These new genes were also found to possess putative consensus AflR binding sites in their promoters, strengthening the evidence of their control by AflR.

Materials and Methods

Fungal strains and culture conditions.

Conidia (10⁶/mL) of Aspergillus parasiticus (Speare) strain SU1 (ATCC 56775) and a ΔaflR derivative of SU1 (courtesy of Dr. Jeff Cary, USDA-ARS, SRRC) were inoculated into 1 L flasks consisting of 400 mL YES broth (2% yeast extract, 5% sucrose, pH 4.5) plus 0.4% agar. The addition of agar maintained confluent growth in submerged culture. Seed cultures were grown in shake culture (200 rpm) for 16 hours at 28°C. Twenty milliliter aliquots of the seed cultures were used to inoculate daughter cultures (200 mL each) of SLS broth (85 g/L sucrose, 10 g/L asparagine, 50 mL/L 20x Salts [70 g/L (NH₄)₂SO₄, 15 g/L KH₂PO₄, 7 g/L
MgSO₄·7H₂O, 1.5 g/L CaCl₂·2H₂O, 0.4 g/L ZnSO₄·7H₂O, 0.1 g/L MnCl₂·4H₂O, 0.04 g/L (NH₄)₆Mo₇O₂₄·4H₂O, 0.04 g/L Na₂B₄O₇, 0.04 g/L FeSO₄·7H₂O). The daughter cultures were then incubated at 28°C, 200 rpm for 8, 16 or 24 hrs. Five cultures were inoculated per strain for the 8 hr timepoint, two cultures per strain for the 16 hr timepoint, and one culture per strain for the 24 hr timepoint in order to procure sufficient tissue for RNA extraction. Two biological replications of the experiment were performed, with the resulting data analyzed together.

**RNA preparation.**

Tissue handling and RNA preparation were performed as previously described [11], with the following exceptions: after isolation of the total RNA with Trizol, the RNA was subjected to an overnight precipitation in 2 M LiCl. After precipitation, the RNA was pelleted in a microfuge and washed once with 75% ethanol. The RNA was centrifuged again, and allowed to air dry for 20 min. Finally, the RNA pellet was resuspended in 50 µL DEPC-dH₂O with 40 units RNasin™ RNase inhibitor (Promega Corporation, Madison, WI) and quantified by spectrophotometry. RNA preparations were visualized by gel electrophoresis to ensure quality.

**Microarray configuration and target labeling.**

The microarrays used in this study were obtained from The Institute for Genome Research (TIGR). PCR products were generated from EST clones belonging to the Southern Regional Research Center, ARS-USDA and spotted onto glass slides. A total of 5002 genes were arrayed at least 3 times each for a total of 29,952 spots. Total RNA from each treatment studied was converted to cDNA and labeled as previously described [11].

**Microarray hybridization, data acquisition, and analysis.**

Comparisons were made between treatments using a loop design (Figure 1). Using this design, in silico comparisons can be made between each node in the loop. Furthermore, each treatment is labeled with each dye, removing effects on measurements caused by the individual dyes. The hybridized slides were scanned using a Perkin Elmer ScanArray
Figure 1. Loop design for microarray analysis of the ΔaflR strain vs. wild type.
Comparisons were made between strains at each timepoint. The construction of this loop allows for in silico comparisons between any two treatments.

Express Lite scanner (Perkin Elmer Life and Analytical Sciences Inc., Boston, MA). Spot intensity data was extracted from the images using UCSF-Spot [24]. The resulting spot-intensity data was then analyzed using the mixed procedure in SAS (SAS v8, SAS Institute, Cary, NC) as described previously [11]. Briefly, least squares estimates of gene-specific treatment effects between pairs of treatments were obtained for each gene under each treatment condition. Differences between treatment effects (least squares estimates) for pairs of conducive and non-conducive conditions can be considered as log2-transformed fold changes [25]. Genes were designated as significantly different in the individual microarray experiments if they possessed p-values less than α. The value of α (0.003) was chosen based on the minimum significance value for the AF pathway genes included on the array.

Results

Transcriptional behavior of aflatoxin pathway genes.

As predicted, no aflatoxin was detected in cultures of the ΔaflR mutant at any of the three time points examined. In contrast, aflatoxin was detected in SU1 cultures at 8 hr and its
concentration increased to a maximum of 2010 ng/ml at 24 hr. (Figure 2). This profile of aflatoxin production was similar to that observed in previous studies [26].

An analysis of gene transcription at each of the three time points using the DNA microarrays revealed 23 genes more highly expressed in SU1 than the ΔaflR derivative of SU1 at every timepoint examined (Figure 3). All but four of these upregulated genes have been shown to be directly involved in aflatoxin biosynthesis or to also reside in the aflatoxin cluster. With the exception of aflR, aflatoxin biosynthetic genes possessing a consensus promoter binding site for Afl (5'-TCGSWNNSCGR-3'; [15]) were upregulated in SU1 (Table 1). aflY, which was listed in the cluster by Yu et al. [27] but not assigned a function, was shown to be upregulated in SU1 and to have a consensus AflR binding site. Several aflatoxin biosynthetic genes without putative AflR binding sites were also upregulated, including aflA, aflJ, aflL, aflO, aflU, aflV, aflW, and aflX. The differences seen in aflatoxin pathway gene expression between timepoints within strain in this study were not as large as the differences observed between strains at a given timepoint (data not shown).

Four genes (hlyC, niiA, hypB, and nadA) also were observed to be upregulated in SU1, and possess consensus AflR binding sites in their promoters (Table 1). Two of these genes are not located near the aflatoxin biosynthetic cluster. One of the genes is nitrite reductase (niiA), which is located in the nitrate assimilation cluster, and is divergently transcribed from the same promoter as niaD, the gene encoding nitrate reductase. niiA possesses a consensus AflR binding site approximately 2.3 kb upstream, within the coding region of niaD (data not shown). The second EST, TC11297, is a homolog of hlyC encoding α-hemolysin from Aeromonas hydrophila, is located approximately 1.5 Mb from the aflatoxin cluster (data not shown) and has a putative AflR binding site approximately 1.8 kb upstream of the putative coding region. hypB is located in the aflatoxin biosynthetic cluster between aflI and aflL (data not shown). This previously undiscovered gene has two putative AflR binding sites located 100 bp and 1.3 kb upstream of the putative coding region. The fourth gene, nadA, was previously described as part of a sugar utilization cluster neighboring the aflatoxin pathway cluster [28]. This gene encodes a putative NADH oxidase. These enzymes can perform the reversible reaction oxidizing NADH to NAD+. A putative AflR binding was identified 1.3 kb upstream of the nadA gene.
Figure 2. Aflatoxin production in cultures of *A. parasiticus* SU1 and Δ*aflR*. Cultures of *A. parasiticus* strains SU1 and Δ*aflR* were grown for 16 hours at 28°C for mycelia production. 20 mL aliquots of these cultures were then inoculated into daughter cultures and incubated for 8, 16 and 24 hours at 28°C, 200 rpm for the production of tissue. RNA was prepared from these tissues for microarray analysis.
Figure 3. Relative expression levels for genes upregulated in *A. parasiticus* SU1. Least square means estimates of expression obtained from the mixed-model analysis of the microarray data (see Methods for explanation and for relationship to fold changes) were plotted for genes upregulated in *A. parasiticus* SU1.
Table 1

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*aThese genes were previously shown or currently determined to possess an AflR binding site in their promoter regions.

Agreement of microarray data with previous results.

The microarrays used in this study shared 324 genes with a targeted microarray used in a previous study [11]. Differences seen by Price and colleagues between conducive and non-conducive treatment pairs were consistent with the differences seen between SU1 and ΔaflR at each timepoint in the present study (Figure 4).
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Figure 4. Hierarchical cluster of aflatoxin pathway genes represented on both the microarrays used in this study and those used by Price et al., 2005. The aflatoxin pathway genes represented on two different microarrays are shown. New gene designations are given, with previous designations in parentheses [27]. 324 genes are shared in total between both microarrays (this study and [11]). Two-way hierarchical clustering of fold changes represented as log₂ measurements was performed (as explained in [11]) for the comparisons shown. Differences between strains at each time were clustered, as were differences between conducive and non-conducive culture treatments.

Seven genes shown to be consistently differentially expressed by Price and colleagues [11] were also differentially expressed in at least one treatment comparison in the present study (Figure 5). Two of these genes are the aflatoxin pathway genes aflM and aflP, which are responsible for mid- and late-pathway enzymatic steps. One gene previously
Figure 5. Comparison of relative expression levels for genes in this study which were differentially expressed by culture conditions in a previous study. Least squared means estimates of expression obtained from the mixed-model analysis of the microarray data (See Methods for explanation and relationship to fold-changes) were plotted for genes differentially expressed in this study and in a previous study [11]. Two aflatoxin pathway genes (CA747496 and CA747926) are included. Conducive aflatoxin conditions in the previous study are 28°C, Sucrose, Ammonium, and pH 4.5. Non-conducive conditions are 37°C, Lactose, Nitrate, and pH 8.0.

demonstrated by Price and colleagues [11] to repress aflatoxin production, CA747470, was upregulated 1.8 fold in SU1 at 24 hours in the present study. In agreement with the observation by Price and colleagues, this gene was generally highly expressed (Figure 5).
Discussion

cDNA microarrays are useful for identifying the consequences of gene alterations (ie. gene-deletion, overexpression) on the transcriptome. We employed an expanded microarray containing 5002 ESTs from a previous EST sequencing project [29] to investigate the possible role of aflR in regulating gene expression outside of the established aflatoxin biosynthetic cluster. We found that the aflatoxin pathway specific transcriptional regulator aflR was able to affect the expression of eighteen of the aflatoxin cluster genes (Table 1), as well as 5 genes not previously proven to be under its influence. Interestingly, three of these genes (hypB, aflY, and nadA) were in or next to the established aflatoxin cluster [27, 30]. The last two genes, hlyC and niiA, were located on separate scaffolds of the A. flavus genome sequence (data not shown).

Of the three genes at or near the aflatoxin pathway locus, aflY and nadA were previously identified [27, 28, 30]. aflY is included in the aflatoxin cluster [30], while nadA is part of the neighboring sugar utilization cluster [28]. In their report of the aflatoxin cluster sequence, Yu and colleagues stated that the genes of the sugar utilization cluster in A. parasiticus did not possess consensus AlfR binding sites, but were likely involved in aflatoxin production due to their inclusion in the EST library. To the contrary, we have found evidence of an AflR binding site approximately 1.25 kb upstream of the nadA coding sequence in A. flavus. Its reduced expression in the ΔaflR strain and similarity of expression to other pathway genes in these experiments (Figure 3) suggests that nadA may in fact belong in the aflatoxin biosynthetic cluster. A role for nadA in aflatoxin biosynthesis is not known. It is possible that A. flavus utilizes nadA, encoding NADH oxidase, to increase reducing power in the cell, and thus increase the available NADH required for many of the aflatoxin biosynthetic enzymes.

Two genes regulated by AflR were located outside of the aflatoxin cluster. One of these genes encodes nitrite reductase, and is part of the well characterized nitrate utilization cluster [31]. Interestingly, a consensus AflR binding site was found approximately 2.3 kb upstream of the coding region of niiA, within the coding region for niaD. Since the ΔaflR strain was made by natural mutation of the niaD locus with chlorate treatment, it is unclear whether the difference in gene expression is due to the mutation of niaD, or the deletion of
aflR. niiA transcript was detected in the ΔaflR strain, suggesting that the minimal niiA promoter is functional. Furthermore, even if the AflR binding site was affected, either altering the AflR site or deleting aflR should yield a similar impact on niiA expression. The prospects for aflR control of nitrite reductase expression, in the light of nitrate repression of aflatoxin production, compel further investigation of this putative interaction.

Finally, the gene expression data obtained in this study were compared to previously reported data investigating the impact of nutritional and environmental factors on the A. parasiticus transcriptome [11]. Of the 5002 ESTs printed on these microarrays, 324 were also printed on the targeted arrays used in the previous study. The aflatoxin genes in common to both arrays were similarly expressed in both studies (Figure 4). Of 17 genes previously reported to be consistently differentially expressed, 7 were present on these arrays and were also differentially expressed in at least one timepoint (Figure 5). Two of these genes are aflatoxin biosynthetic genes (CA747496/aflM and CA747926/aflP). Once again, CA747470 was shown to be highly expressed over all treatments, but unlike the previous experiment, CA747470 was upregulated in the wild type (aflatoxin conducive treatment) rather than in the ΔaflR strain (aflatoxin non-conducive treatment). Overexpression of CA747470 represses aflatoxin production in A. flavus [11].

In conclusion, AflR appears to regulate genes outside of the established aflatoxin cluster. Two of these genes can be theorized to impact aflatoxin production by altering intracellular conditions (nadA and niiA), whereas one gene, hlyC (encoding α-hemolysin), has no apparent role in aflatoxin production but may play a role in pathogenesis. In order to more fully understand the biology of this important fungus, a full genome microarray is needed. Future experiments should focus on deletion of genes identified in this study, as well as transcription profiling of various established mutant lines deficient in aflatoxin production.
References


Rho-GDP dissociation inhibitor affects growth and aflatoxin production in *Aspergillus flavus*.

Michael S. Price and Gary A. Payne*

*North Carolina State University, Center for Integrated Fungal Research, Box 7567, Raleigh, NC 27695-7567.
E-mail: gary_payne@ncsu.edu
Telephone: 919-515-6994
Fax: 919-513-0024.
Abstract

Regulation of aflatoxin (AF) production is complex, involving transcriptional and post-transcriptional regulation focused mainly through the pathway specific transcriptional regulator aflR. An investigation into the nature of the transcriptional regulation of AF production by comparing conducive and non-conducive culture conditions revealed a clade of genes with a similar transcription profile to that of aflR. One of these genes, a putative Rho-GDP dissociation inhibitor, was characterized by gene deletion and shown to regulate AF production in Aspergillus flavus. The protein encoded by this gene, Afrdi1, showed 45% identity to Rdi1p in S. cerevisiae. However, the ΔAfrdi1 mutant phenotype is most similar to the bem4 null mutant in yeast, which exhibits a severe growth defect on minimal medium, a moderate growth defect on complete medium, and a temperature sensitive phenotype. Moreover, the ΔAfrdi1 mutant produces 97.3% less toxin than wild type. Inferences from S. cerevisiae reveal a possible link between AfRdi1 and RasA, which has been shown to regulate sterigmatocystin production in A. nidulans.

Introduction

Aflatoxin (AF), produced by select members of the genus Aspergillus, is the most potent naturally occurring carcinogen known (Squire, 1981). Two of the five Aspergilli known to produce AF are the plant pathogens A. flavus and A. parasiticus. These species are responsible for AF contamination in corn, peanuts, cotton, and certain tree nuts. In addition to losses incurred during food production, post-harvest contamination of food and feed with AF is also a problem. In the US, agricultural economic losses due to AF contamination of food and feed amount to $270 M annually (Richard and Payne, 2003).

Since initial investigations leading to the establishment of aflatoxin as the cause of Turkey X-disease in the 1960’s, AF has become the most studied mycotoxin. The hazards of consuming aflatoxin contaminated food and feed, along with the trade restrictions imposed on aflatoxin-contaminated grain, has prompted a concerted research effort to better understand the ecology of the fungus and to elucidate the biosynthesis and regulation of AF. To date, nearly all of the biosynthetic steps have been characterized, and the DNA sequence encoding the AF pathway genes has been elucidated (Price and Payne, 2004; Yu et al., 2004).
Further, some of the upstream regulatory elements that link development in the fungus with the onset of aflatoxin production have been characterized (Calvo et al., 2002). Recently, a putative global regulator of secondary metabolism, \textit{laeA}, was identified (Bok and Keller, 2004). The regulatory mechanism of this gene on aflatoxin biosynthesis in the context of other secondary metabolic pathways is not known. It is becoming clear that aflatoxin biosynthesis is regulated by a complex network and that many elements of the network remain to be characterized.

Recently, a targeted cDNA microarray was developed and used to monitor gene transcription associated with aflatoxin production (OBrian et al., 2003). The 753 elements on the array were from ESTs sequenced from a cDNA library constructed from cultures producing aflatoxin. The array was used to probe transcripts of cultures differing in nitrogen source, carbon source, growth temperature or pH. Analysis of gene expression in cultures grown in conditions conducive or nonconductive for aflatoxin production revealed a homolog (\textit{Afrdi1}) of the Rho GDP-dissociation inhibitor (GDI) \textit{rdi1} from \textit{S. cerevisiae} that shared an expression pattern with the AF pathway-specific transcriptional regulator \textit{aflR}.

In yeast, Rdi1p interacts with the GTPases Rho1p and Cdc42p, and inactivates them by sequestration of these proteins in the cytosol (Koch et al., 1997). Rho1p is a member of the small G-protein superfamily, and is involved in actin polymerization and polarized growth (Guest et al., 2004). Cdc42p is another member of the G-protein superfamily, and is involved in the establishment and maintenance of cell polarity in yeast (Johnson, 1999). Both of these proteins initiate signal transduction cascades leading to gene transcription, and interact with the GDI-like protein Bem4p.

In order to investigate a possible role for \textit{Afrdi1} in AF production, we deleted the gene from \textit{A. flavus} and assessed fungal growth and AF production in the resulting mutant. The results presented here suggest that \textit{Afrdi1} plays a role in hyphal growth and branching in \textit{A. flavus}, and may be involved in regulating AF production via an intracellular signaling cascade.
Materials and Methods

Identification of the *Afrdi1* coding region. The Universal GenomeWalker™ kit (BD Biosciences Clontech, Palo Alto, CA) was used to determine the genomic sequence flanking EST CA747847 (*Afrdi1*). The genomic sequence data were analyzed by the FGenesH gene prediction software package* using “Aspergillus” as the organism.

Fungal transformation and screening. *Aspergillus flavus* 3357-5, a pyrG mutant of NRRL 3357 (USDA/ARS/NCAUR), was used as the recipient strain for the gene disruption construct of *Afrdi1*. Cultures were maintained on Potato Dextrose Agar (PDA; Becton, Dickinson and Co., Sparks, MD) with 10 mM uracil where appropriate. Fungal protoplasts were transformed using a PEG-based procedure (Woloshuk et al., 1989). Disruption constructs were made using a PCR-based method (Davidson et al., 2002). Primers for amplifying 1 kb regions flanking *Afrdi1* and the selectable marker *pyr4* from *Neurospora crassa* are listed in Table 1.

<table>
<thead>
<tr>
<th>Primer #</th>
<th>Sequence (5’-3’)</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCGACGTACTGTATCAGTTCCG</td>
<td>5’ flank</td>
</tr>
<tr>
<td>2</td>
<td>CTTTACCGTATGCAAGAACTTGAGACCACACGAGTCAAG</td>
<td><em>pyr4</em></td>
</tr>
<tr>
<td>3</td>
<td>CTTGACTCTGTGTGCAAGTCTTGATCACAACGAGGTAAGG</td>
<td>5’ flank</td>
</tr>
<tr>
<td>4</td>
<td>GCCGGAGGGATATTTTCGGTTTTTTCTTTTTTCAGTCCAAGAGGAAGA</td>
<td>3’ flank</td>
</tr>
<tr>
<td>5</td>
<td>TCTTCCCTCTGGACTGAAAGAAAACGAAATCTCTCCTCCGCC</td>
<td><em>pyr4</em></td>
</tr>
<tr>
<td>6</td>
<td>AAGTTACGATACCCCCATCG</td>
<td>3’ flank</td>
</tr>
</tbody>
</table>

Transformants able to grow in the absence of uracil were transferred to PDA plates for PCR screening. Primers 1 & 6 from the disruption construct (Figure 1) were used to verify gene replacement in the fungal transformants. Presence of a single 3.7 kb PCR product in the transformants signified the deletion of CA747847.

Characterization of the *A. flavus rdi1* deletion strain (Δ*Afrdi1*). In the initial characterization, the Δ*Afrdi1* mutant was grown on Czapek-Dox Broth minimal medium plates (MM; Becton, Dickinson and Co., Sparks, MD) amended with either 0.5M KCl, 1M

* http://www.softberry.com
Figure 1. Construction of linear gene-deletion construct for CA747847. PCR primers were synthesized to amplify the 5' and 3' flanking regions surrounding the putative CA747847 coding region. Internal primers were synthesized as hybrid sequences between the 5' or 3' region and the pyr4 marker gene from N. crassa. The final construct is produced during a PCR reaction utilizing the products of PCR #1 as template, and the forward and reverse primers 1 & 6.

KCl, 0.5mM CuSO₄, 5mM CuSO₄, pH 3, or pH 8. Growth on the six media was compared to that of the parental strain, 3357-5.

To assess the effect of temperature on the ΔAfrdi1 mutant, its growth at 28°C and 37°C was compared to that of 3357-5, which contains a wild type copy of the gene. Triplicate cultures of each strain were grown in petri dishes on either MM, MLS (MM + 0.4 M (NH₄)₂SO₄), or PDA solid medium. Crosswise measurements were taken from each plate.
daily and averaged according to strain, media, and temperature. To assess AF production in
the deletion strain, 10\(^6\) conidia per mL culture of \(\Delta Afrdi1\) and 3357-5 were grown in PBD
(Potato Dextrose Broth; Becton Dickinson & Co., Sparks, MD) at 37°C, 200 rpm for 24 hrs
for sufficient biomass production. The cultures were then shifted to 28°C, 200 rpm for 24
hrs for the production of AF. Aflatoxin was quantified by HPLC. Duplicate cultures for
each strain were grown.

The morphology of the \(Afrdi1\) mutant was compared to the wild type strain by
examination of the two strains under a compound microscope at 20X and 40X. The two
strains were grown at 28°C and 37°C on a cellophane membrane overlayed onto either MM
or PDA medium. After 24 hrs, the cellophane membranes were moved to MM or PDA plates
containing 10 mM uracil and incubated a further 24 hrs to allow the growth of 3357-5.
Squares were then cut from each colony, mounted on microscope slides, and stained with
lactophenol blue. The images presented are typical for each strain/media/temperature set
examined.

Results and Discussion

Characterization of \(Afrdi1\) in \(A. flavus\). In a series of microarray experiments we
found that a gene corresponding to EST CA747847 followed the same expression pattern as
of the AF pathway-specific regulatory gene \(aflR\) (data not shown). Because of its expression
pattern we reasoned that it would be a good candidate for functional analysis to determine if
it is involved in aflatoxin biosynthesis. The full length gene sequence corresponding to the
EST was obtained using the GenomeWalker\textsuperscript{TM} kit. This gene exists as a single copy within
the \(A. flavus\) genome. Sequence comparisons of the predicted protein from CA747847 with
the translated nucleotide database at NCBI revealed 45% identity to \(rdi1\) in \(S. cerevisiae\).

Gene deletion strategy for \(Afrdi1\). To determine the function of this gene in \(A. flavus\) it was deleted with a linear PCR-created gene-deletion construct containing 1000 bp of
flanking sequence surrounding the predicted \(Afrdi1\) gene. This PCR-based cloning strategy
Figure 2. PCR screen of ΔAfrdi1 gene-deletion mutants. Transformants exhibiting prototrophic growth on medium lacking uracil were analyzed by PCR to identify those containing the deletion construct. The predicted size of the deletion amplicon is 3.7 kb and the wild type amplicon is 2.9 kb. 13 out of 45 transformants are shown. One transformant out of forty-five was observed to possess the gene deletion.

has been used successfully in Cryptococcus neoformans (Davidson et al., 2002). PCR primers were developed to replace the entire putative coding region of Afrdi1 with the pyr4 gene from N. crassa (Figure 1).

Transformant colonies were screened by PCR using primers 1 and 6 (Table 1) to identify those transformants with the gene deletion. As shown in figure 2, one transformant exhibited a single band approximately 3.7 kb in size corresponding to the size of the deletion construct (Lane 8). One other transformant also exhibited the 3.7 kb band, but retained the 2.9 kb native band indicating no replacement at this locus had taken place.

Characterization of the Afrdi1 deletion in A. flavus. In yeast, the protein encoded by rdi1 is involved in actin filament organization by interacting with Rho1p. Rho1p is a member of the small G-protein superfamily of signaling proteins, and is itself involved in actin polymerization and organization. Rdi1p regulates the activity of Rho1p by binding the
GDP-bound form of Rho1p and removing it from the membrane and into the cytosol (Koch et al., 1997).

Yeast mutated at the \textit{rdil} locus exhibit moderate growth defects on minimal medium and yeast peptone dextrose medium. As shown in figure 3, growth of the $\Delta$Afr\textit{rdi1} strain was severely inhibited at 28°C on minimal media in all treatments. Since 28°C is the optimal temperature for aflatoxin production but not for growth of \textit{A. flavus}, we repeated this experiment using MM, MLS, and PDA at either 28°C or 37°C. As shown in figure 4, growth on MM and MLS at 28°C was again severely repressed, while PDA was able to ameliorate the effect of the \textit{rdi1} deletion. At 37°C, growth on MM was still repressed but not to the same extent, and growth on MLS and PDA were much improved. Growth on PDA at either temperature was luxurious, and abundant conidia were produced.

The morphology of $\Delta$Afr\textit{rdi1} was observed microscopically (Figure 5). The mutant was able to germinate on MM at 28°C, but hyphal growth was limited even after 48 hrs (Fig 5, panel A). In contrast, the mutant grew as well as wild type at 28°C on PDA (Figure 5, panel C). Furthermore, the $\Delta$Afr\textit{rdi1} mutant exhibited wild type morphology on both MM and PDA when grown at 37°C (Figure 5, panels E and G), however growth was always slower than for 3357-5 (Figure 4). Growth at 37°C was observed to be more dense for both strains than at 28°C, especially on PDA (Figure 4).

Differences were observed for spacing of hyphal branches between $\Delta$Afr\textit{rdi1} and 3357-5, as exemplified at 28°C on PDA (Figure 5, panels C and D). For the same length of mycelium, 3357-5 produces three hyphal branches whereas $\Delta$Afr\textit{rdi1} only produces two (black arrows). Furthermore, the cells were approximately 70% longer in the mutant than the wild type strain, apparently due to fewer septations (Figure 5, panels G and H). These differences in hyphal branching and septation may be due to the inability of the $\Delta$Afr\textit{rdi1} mutant to correctly coordinate its actin cytoskeleton. The targets of Rdi1p in yeast are Rho1p and Cdc42p, both of which belong to the small G-protein superfamily. Cdc42p is involved in bud site selection and asymmetrical distribution of actin structures in yeast (Pruyne and Bretscher, 2000; Zhang et al., 2001). Rho1p organizes at sites of bud emergence in yeast and
**Figure 3. Growth of ΔAfrdi1 on minimal media with different amendments.** The ability of the ΔAfrdi1 strain to grow on minimal media, and in the presence of salt, copper and high and low pH was observed. Plates were inoculated with spores by toothpick and incubated for 5 days at 28°C. Radial growth presented is the mean of crosswise measurements taken from 3 replicate plates.
Figure 4. Growth of ΔAfri1 on MM, MLS, and PDA at either 28°C or 37°C. Spores of each strain were inoculated onto plates of MM, MLS, or PDA by toothpick. The plates were incubated at either 28°C or 37°C for 5 days. Measurements were taken as described in Figure 3.
Figure 5. Growth comparison of ΔAfrdi1 and 3357-5 on MM and PDA. ΔAfrdi1 and 3357-5 spores were inoculated onto cellophane on either MM and PDA. The plates were incubated at 28°C or 37°C for 24 hrs. The cellophane from each plate was then moved to either MM or PDA containing 10 mM uracil to allow the growth of 3357-5 and incubated for 24 hrs at either 28°C or 37°C. Panel A - Δafrdi1 on MM at 28°C (40X magnification); panel B - 3357-5 on MM at 28°C (40X magnification); panel C - Δafrdi1 on PDA at 28°C (40X magnification); panel D - 3357-5 on PDA at 28°C (40X magnification); panel E - Δafrdi1 on MM at 37°C (40X magnification); panel F - 3357-5 on MM at 37°C (40X magnification); panel G - Δafrdi1 on PDA at 37°C (20X magnification); panel H - 3357-5 on PDA at 37°C (20X magnification). Bar represents 100 µm. Arrows indicate differences in hyphal branching between strains.
coordinates the localization and activity of a number of genes involved in cell wall production and actin polymerization (Madden and Snyder, 1998). Dominant active mutants of the homolog of this gene in A. nidulans, rhoA, exhibit altered hyphal branching, growth, and cell wall deposition (Guest et al., 2004). Dominant active mutants of rhoA should be similar to the Afrdi1 deletion, as AfRdi1 should serve to inhibit the activity of RhoA in A. flavus.

Because transcription of ΔAfrdi1 follows the same pattern as for the aflatoxin pathway regulatory gene, aflR, we predicted that this gene may be involved in aflatoxin biosynthesis. To examine this further, the wild type and mutant strain were grown under conditions conducive for aflatoxin production. As stated earlier, the ΔAfrdi1 mutant does not grow well at 28°C, the optimum temperature for aflatoxin production. To ensure that both cultures started with similar biomass, we used a temperature shift protocol. The cultures were grown for 24 hrs at 37°C in PDB medium to allow for growth, then shifted to 28°C for the production of aflatoxin. As shown in table 2, the ΔAfrdi1 mutant produced far less aflatoxin (2.7%) than 3357-5 on a mass basis.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aflatoxin (µg/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3357-5</td>
<td>20067</td>
</tr>
<tr>
<td>Afrdi1</td>
<td>539</td>
</tr>
</tbody>
</table>

*Both mutant and wild type were grown @ 37°C for 24 hrs for biomass production, then shifted to 28°C for 24 hrs for aflatoxin production.*

Sequence analysis of Afrdi1 showed homology to rdil in yeast, and the rdil null yeast strain exhibits a growth defect on minimal media, as does the ΔAfrdi1 mutant. However, the phenotypes we observed are not exactly like those reported for rdil in yeast. They more closely resemble the predicted phenotypes observed for the null mutation of bem4, a gene involved in bud emergence and establishment of cell polarity in yeast (Mack et al., 1996). The null mutant of bem4 exhibits a severe growth defect on minimal media, as well as a moderate growth defect on yeast peptone dextrose medium, and also shows a cold-
and temperature-sensitive growth phenotype*. Bem4p binds Rho1p and Cdc42p, and sequesters them to the cytosol, as does Rdi1p in yeast. A search of the genome sequence of *A. flavus* revealed no homolog of *bem4*.

A tenable hypothesis to explain our findings is that *AfRdi1* also plays the role of *bem4* in *A. flavus*. If it is true that *AfRdi1* is functionally homologous to *bem4*, then the regulatory network leading to AF production begins to emerge. In yeast, Bem4p interacts with a protein

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**Figure 6. Hypothetical gene interaction network in *A. flavus* for control of AF biosynthesis.** The *bem4/rdi1* gene in *A. flavus*, *AfRdi1*, may control AF production in *A. flavus* by modulating the activity of Bbc1, which in turn would affect the activity of RasA in the fungus. RasA inhibits transcription and activity of *aflR* and AflR, respectively, in *A. nidulans* (Shimizu et al., 2003).

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* http://www.yeastgenome.org
responsible for actin cytoskeleton organization and biogenesis named Bbc1p (Tong et al., 2001). Bbc1p interacts with Ras2p in yeast (Tong et al., 2001). This is important, as the homolog of ras2 in A. nidulans (rasA) regulates aflR at both the transcriptional and post-transcriptional levels (Shimizu et al., 2003). Furthermore, tBLASTn analysis of the A. flavus genome with the protein sequence of Bbc1p from yeast identifies a single homolog of bbc1 in A. flavus (data not shown).

It is unknown what type of interaction exists between Bem4p and Bbc1p, or between Bbc1p and Ras2p in yeast. If these interactions hold true in A. flavus, then the network of these interactions can be explained as in Figure 6. AfRdi1 putatively inhibits the activity of RhoA and Cdc42, leading to polarized growth and proper hyphal branching. AfRdi1 also putatively inhibits the activity of Bbc1. Bbc1 putatively activates RasA, which putatively inhibits AF production (Shimizu et al., 2003). If this is true, then deletion of the AfRdi1 gene in A. flavus should produce an altered growth phenotype and repressed AF production, which are the observed phenotypes presented in this report.

These data implicate a putative GDP-dissociation inhibitor, AfRdi1, in the control of AF biosynthesis in A. flavus. This gene is phenotypically similar to bem4, which lacks a homolog in A. flavus, and can be linked to the AF-controlling protein RasA. Future studies looking into the ability of bem4 from yeast to alleviate the cold-sensitive phenotype of AfRdi1 are warranted, as is the ability of rdi1 from yeast to complement the defects reported in this study. Investigations into the ability of bbc1 to influence AF production are also appropriate. We hypothesize that the deletion of bbc1 in A. flavus may disrupt cell signaling leading to the production of aflatoxin. More work needs to be done to confirm a role for this signaling cascade in regulation of aflatoxin production.
References


