ANALYSIS OF A MYCOTOXIN GENE CLUSTER IN *ASPERGILLUS NIDULANS*

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Abstract.
Aspergillus nidulans has functioned as a model system for the study of fungal genetics since the 1950s. Application of methodologies ranging from Mendelian genetics to the most sophisticated molecular biological techniques have resulted in a detailed understanding of genes and pathways involved in primary metabolism, secondary metabolism and development in *A. nidulans*. We have taken advantage of this background in developing *A. nidulans* as a genetic system to study the molecular mechanisms regulating aflatoxin biosynthesis. Aflatoxin, a carcinogenic polyketide, is the product of a lengthy biochemical pathway found in the asexual spp., *A. flavus* and *A. parasiticus*. *A. nidulans* possesses most if not all of this pathway and produces sterigmatocystin, the penultimate precursor of the aflatoxin pathway. We have identified a ~60 kb cluster of genes in *A. nidulans* whose products are involved in sterigmatocystin biosynthesis. This cluster contains at least 20 genes proposed to encode both enzymatic activities and regulatory proteins. Our results have shown that at least some of these genes are functionally conserved between *A. nidulans*, *A. flavus* and *A. parasiticus*, and that they are regulated in similar ways. Further studies of sterigmatocystin regulation in *A. nidulans* should yield information transferable to studies of (i) secondary metabolism in other filamentous fungi and (ii) aflatoxin regulation in *A. flavus* and *A. parasiticus* in particular.

Introduction

Aspergillus spp. constitute a major portion of the mycotoxin producing fungi that contaminate US and world food supplies. Several species, including *A. flavus*, *A. nidulans* and *A. parasiticus* produce one or both of the toxic and carcinogenic metabolites aflatoxin (AF) and sterigmatocystin (ST) (Aucamp and Holzapfel, 1970; Bennett and Christensen, 1983; Hajjar et al., 1989). AF and ST are products of the same biosynthetic pathway (**Figure 1**) and these compounds are found contaminating diverse foods including peanuts, corn, tree nuts, cheese, milk and meat (Bullerman et al., 1969; Jelinek et al., 1989; Northolt et al., 1980). A compilation of epidemiological studies, feeding studies, biochemical and chemical studies have established that both compounds cause mammalian hepatocarcinomas (Adamson, 1991; Fujii et al., 1976) and are classified as carcinogens (Adamson, 1991; Fujii et al., 1976; Mori, 1992; Xei, 1990; Wogan, 1992). Consequently, strict national and international standards of allowable amounts of AF (more commonly analyzed than ST levels) in food supplies have been imposed to protect public health. These standards have traditionally been met by destroying contaminated food products or by decontamination using ammoniation (Park et al., 1988) or aluminosilicate sorbtion (Phillips et al., 1990). These decontamination methods are used mainly for animal feeds while contaminated human food products are generally destroyed or repackaged as feeds.

The longterm goal of the research in our laboratories is to provide alternative methods for meeting the stringent standards imposed on food products by identifying novel means for controlling fungal AF and ST

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Pathway Intermediates	Proposed Activities	Gene in Cluster
Acetate		
v	PKS FAS	Genes 1, 10 and 11
Polyketide Progenitor		
v		
Norsolorinic Acid		
v	Reduction (Dehydrogenation?)	Gene 1 Gene 13 or 19
Averantin		
v		
Averufanin	Oxidation	Gene 2,6,7,12 or 20
v		
Averufin		
v	Oxidation	Gene 2,6,7,12 or 20
1-Hydroxyversicolorone		
v	BV Oxidation	Gene 2,6,7,12 or 20
Versiconal Hemiacetal Acetate		
v	Esterase	Gene 9
Versiconal		
v	Oxidation or Dehydration	Gene 2,6,7,12 or 20 Gene 13 or 19
Versicolorin B		
v	Desaturation (Oxidation)	Gene 2,6,7,12 or 20
Versicolorin A		
v	BV Oxidation NADPH Ketoreduction Decarboxylation	Gene 16 (*verB*) Gene 18 (*verA*) ?
Demethylsterigmatocystin		
v	Methylation	Gene 13
<u>Sterigmatocystin</u>		
v	Methylation	Absent in *A. nidulans*
o-methyl Sterigmatocystin		
v	Dioxygenation	
Aflatoxin B_1		

Figure 1. The Aflatoxin Biosynthetic Pathway. Proposed enzyme activities and genes in the sterigmatocystin cluster (see Figure 4) putatively encoding these activities.

production. We hope to achieve this goal through understanding how AF/ST production is regulated in fungi. We started this work by isolating and characterizing genes required for AF/ST production to begin to understand the molecular genetic processes controlling their expression. Eventually, we hope to elucidate measures to suppress AF/ST biosynthesis in nature. We have used *A. nidulans* to identify the regulatory mechanisms controlling ST biosynthesis to take advantage of the sophisticated molecular genetic approaches available in this experimentally tractable *Aspergillus* spp. (Timberlake, 1990). This paper

will focus on recent progress made in understanding the genetics of ST biosynthesis in *A. nidulans*.

(i) Sterigmatocystin is a polyketide. Aflatoxin and sterigmatocystin are biochemically classified as polyketides. Polyketides are formed by the condensation of acetyl CoA and malonyl CoA by a polyketide synthase (PKS) in a manner similar to fatty acid synthesis by a fatty acid synthase (FAS) (Hopwood and Sherman; 1990, Herbert, 1989). Other notable polyketides include phytotoxins (Kono et al., 1981; Stoessl, 1981), spore pigments (Bell and Wheeler, 1986; Brown et al., 1993; Mayorga and Timberlake, 1992) and antibiotics (Katz and Donadio, 1993). Initial progress in elucidating the ST/AF biosynthetic pathway was made through the use of *Aspergillus* mutants blocked in AF production, radiolabeled precursor feedings, enzyme inhibitor studies, and biochemical characterization of enzymatic activities (for review articles see Bennett and Christensen, 1983; Bhatnagar et al. 1992; Dutton, 1988; Herbert, 1989). There remains some debate as to whether the ST/AF PKS utilizes acetyl CoA or hexanoyl CoA as a starter unit (Chandler and Simpson, 1987; Townsend et al., 1984). In the first case, a single decaketide synthase is involved while in the latter case, a hexanoate starter would be synthesized via a FAS and used by an octaketide synthase to construct the ST/AF polyketide precursor.

It is unclear how many enzymatic activities are needed to modify the polyketide precursor to produce ST and AF. Up to 16 enzymatic activities have been proposed to produce AFB_1 (**Figure 1**, Bhatnagar et al., 1992). The molecular rearrangements needed to convert each intermediate to the next known intermediate in the pathway are often complex and it is possible that more than one enzyme is required for each step (Bhatnagar et al., 1992), or that an enzyme has dual functions (Matsushima et al., 1994 and references therein). Purification of enzymes in the pathway has proved difficult since they are often in low abundance and generally short lived (Bhatnagar et al., 1988: Dutton, 1988). Only one enzyme in the AF pathway, a methyltransferase necessary for the conversion of ST to *o*-methylsterigmatocystin, has been purified and used to identify the encoding gene (Keller et al., 1993: Yu et al., 1993). Our progress with cloning genes in the ST gene cluster of *A. nidulans* suggests that identification of all of the pathway enzymes may be best elucidated through characterization of the genes involved in the pathway.

Characterization of the Sterigmatocystin Gene Cluster.

(i) Genes encoding enzymatic functions.

verA. The first ST pathway gene to be characterized in *A. nidulans* was *verA*, a keto-reductase needed to convert versicolorin A to ST (Keller et al., 1994). This gene was located by using the *A. parasiticus* homolog, *ver-1* (Skory et al., 1992), to screen an *A. nidulans* cosmid library (*ver-1* was identified by complementing an *A. parasiticus* AF⁻ mutant which accumulated versicolorin A with a genomic library made from a wildtype isolate of *A. parasiticus*). Cosmid pL24BO3 was identified as containing the *ver-1* homolog, *verA*. A 6.6 kb subclone of pL24B03, pNK10, was found to contain the coding and promoter region of *verA*. Sequence analysis of *verA* showed that the gene products of *verA* and *ver-1* shared 85% identity including a conserved motif indicative of a NADPH binding site (**Figure 2**). This supported earlier biochemical work which demonstrated that the chemical chlobenthiozone, thought to interfere with ketoreduction, blocked the AF pathway at versicolorin A (Wheeler et al., 1991). Disruption of the *A. nidulans verA* gene resulted in a versicolorin A-accumulating strain of *A. nidulans* and formally proved that *verA* (and hence *ver-1*) was required for ST biosynthesis.

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                         1                                                50
A. nidulans verA        mssSDNyRLD GKVALVTGAG RGIGAAIAVA LGqpGAKVVv nyansreaae
A. parasiticus ver1     ..mSDNhRLD GKVALVTGAG RGIGAAIAVA LGerGAKVV. ..........

                        51                                               100
A. nidulans verA        kvvdeiksna qsaisiqADV GDPDAvtKLM dqaVeHFGYL DIVSSNAGIV
A. parasiticus ver1     .......... .......ADV GDPEAtaKLM aetVpHFGYL DIVSSNAGIV

                        101                                              150
A. nidulans verA        SFGHvKDVTP DeFdrvfrvN TR......Gq fFVAreaYR. ...hlrEGGR
A. parasiticus ver1     SFGHlKDVTP EaYa...keN TRsltgssGs tLVAsssWRg rlspyaEGGR

                        151                                              200
A. nidulans verA        IILTSSNTAs vKGVPrHAVY SGSKGAIDTF VRCLAIDCGD KKITVNAVAP
A. parasiticus ver1     IILTSSNTAw lKGVPkHAVY SGSKGAIDTF VRCMAIDCGD KKITVNAVAP

                        201                                              250
A. nidulans verA        GAIKTDMFLs VSREYIPNGE TFTDEQVDEc a......... ..........
A. parasiticus ver1     GAIKTDMFLa VSREYIPNGE TFTDEQVDEv sfppincvll gsrltksylv

                        251                                         295
A. nidulans verA        AWLSPLNRV GLPVDVARVV SFLASDaAEW ISGKIIGVDG GAFR
A. parasiticus ver1     AWLSPLNRV GLPVDVARVV SFLASDtAEW VSGKIIGVDG GAFR
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Figure 2. Sequence alignment of *A. nidulans* VerA and *A. parasiticus* Ver1. The NADPH binding motif is indicated in bold. The GenBank accession number for *A. nidulans verA* is L27325..

<u>*verB*.</u> The VerA ketoreductase is not the only enzyme activity necessary for the conversion of versicolorin A to ST. Bhatnagar et al. (1992) have predicted that this conversion requires an oxidation, lactone cleavage, keto-reduction, oxidative decarboxylation and methylation. We had found four open reading frames (ORFs) in the DNA region surrounding *verA* and were interested in determining whether any of these encoded an enzyme carrying out one of these activities. Using the same strategy as previously used for the *verA* disruption, we have disrupted ORF2 (Brown et al., 1994). This gene, which we call *verB*, is also necessary for the conversion of versicolorin A to ST (Brown et al., 1994). In fact, feeding studies with AF pathway intermediates indicate that both *verA* and *verB* disruptions share the same phenotype, e.g. no ST production but, instead, an accumulation of versicolorin A (Keller et al., in preparation). *verB* shares homologies with several cytochrome p450 monooxygenases (Keller et al. 1994). We suggest that VerB is responsible for the oxidation activity as predicted by Bhatnagar et al. (1992).

<u>Polyketide synthases and fatty acid synthases.</u> As mentioned earlier, the initial step in ST biosynthesis is dependent on a polyketide synthase (PKS) and possibly a fatty acid synthase (FAS). The proteins involved in polyketide and fatty acid synthesis share a significant degree of homology within domains required for specific catalytic activities (Hopwood and Sherman, 1990; Katz and Donadio, 1993). Two of these domains, a ß-ketoacyl ACP synthase (KS) and a acyltransferase (AT), are found in both PKSs and FASs. Using a computer program, conserved amino acid sequences were compared in these domains. Two sets of oligonucleotide primers were designed to the KS domain and one set of primers to the AT domain (**Figure 3**).

Organism and Gene	Conserved Amino Acids Region 1	Region 2
Chicken FAS	GMGTQWKGMG	GILGHSVGELA
Rat FAS	GMGTQWRGMG	GIIGHSLGEVA
Saccharopolyspora erythraea Mod 1	GQGWQWAGMA	AVIGHSQGEIA
Saccharopolyspora erythraea Mod 4	GQGAQWVGMA	AVVGHSQGEIA
Saccharopolyspora erythraea Mod 2	GQGAQWEGMA	AVIGHSQGEIA
Mycobacterium leprae PKS C	GRGSQWAGMG	LVIGHSLGEVA
Mycobacterium leprae PKS D	GRGSQWAGMG	AVIGHSMGEVA
Mycobacterium leprae PKS E	GQGSQWAGMG	AVIGHSMGEVS
Mycobacterium tuberculosis MAS	GQGSQWAAMG	AVVGHSMGESA
Penicillin patulum MSAS	GHGAQWPDMG	AVIGHSVGEIA
Mycobacterium leprae PKS F	GQGAQHVGMA	AYIGYSTGEYI
Aspergillus nidulans wA	GQGAQYAAMG	FVLGHSLGDFA
Escherichia coli cyclopropane FAS	GQGSQTVGML	MMAGHSLGEYS
Consensus sequence:	G-G-QW-GM-	-V-GHS-GE-A
Amino acid sequence for Primers:	GQGSQWAGMA	AVIGHSLGEIA

<u>Region 1 Primer:</u> <u>Region 2 Primer:</u>

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            T       G       C                 G   C           A  CT G        G
GGI CAI GGI GCI CAA TGG GTI GGI AUG G GC AAT TTC ICC IAG IGA ATG ICC AAT IAC IG
                                                                             T
```

Figure 3. Consensus sequence for the acyltransferase (AT) domain of PKSs and FASs. Two areas of conserved amino acid sequences, labeled Region 1 and Region 2 were identified in the 13 listed PKSs and FASs. A consensus sequence was identified for each region and then an amino acid sequence designed from this region. A degenerate DNA primer was designed to the amino acid sequence of each region. The DNA primer of region 1 had 28 nucleotides was 8 fold degenerate and contained 6 inosines. The region 2 primer had 32 nucleotides was 192 fold degenerate and contained 6 inosines. Arrows designate 5' to 3' direction.

The KS and AT primers were used in several different PCR strategies to obtain products. Templates included *A. nidulans* genomic DNA as well as a number of cosmids, including pL24B03, that had previously been shown to contain genes whose products are involved in the biosynthesis of sterigmatocystin (Keller et al., 1994 and Keller et al., unpublished data). Our primer design strategy was successful in identifying a PKS on cosmid pL11C09 (which overlaps pL24B03) and a FAS fragment from *A. nidulans* genomic DNA. Sequence analysis of these PCR products strongly suggests that we have cloned a PKS involved in ST biosynthesis as well as an *A. nidulans* FAS (Brown et. al., unpublished data).

(ii) A gene encoding a regulatory protein. Recently an *A. flavus* AF^- strain, thought to be mutated in a regulatory gene in the AF pathway (Papa, 1979), was complemented by *aflR*, an *A. flavus* gene predicted to encode a binuclear zinc cluster DNA binding protein (Payne et al., 1993). The *A. parasiticus* homolog was found to be identical to *A. flavus aflR* (Chang et al., 1993). The zinc cluster containing the binuclear zinc motif, $CX_2CX_6CX_6CX_2CX_6CX_2$,

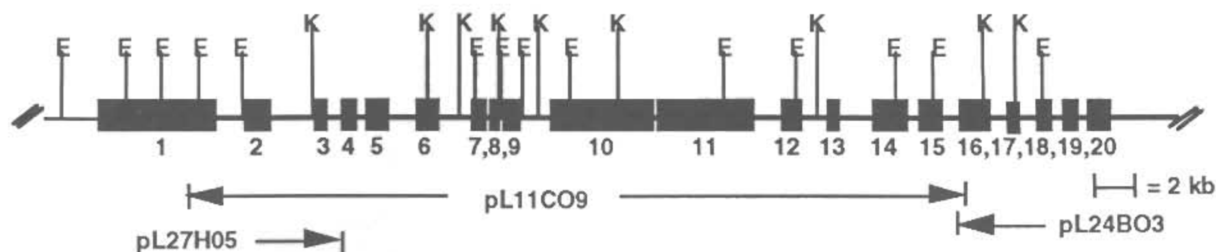

Figure 4. The sterigmatocystin gene cluster in *Aspergillus nidulans*. DNA sequencing and northern analysis of the two overlapping cosmids pL24B03 and pL11C09 have identified 20 transcribed open reading frames (ORFS) bearing homologies to proteins that could provide enzymatic activities or regulatory activities involved in ST biosynthesis. In order from left to right, the 20 ORFs show homology to (1) a polyketide synthase, (2) a monooxygenase, (3) undetermined (4) norsolorinic acid reductase, (5) a zinc binuclear cluster protein, *anaflR*, (6) a monooxygenase, (7) a monooxygenase, (8) undetermined, (9) a carboxyesterase, (10) an a fatty acid synthase, (11) a B fatty acid synthase, (12) a monooxygenase, (13) an alcohol dehydrogease, (14) an *o*-methyltransferase, (15) undetermined, (16) a p450 monooxygenase, *verB*, (17) the gamma subunit of elongation factor one, (18) a NADPH-dependent ketoreductase, *verA*, (19) an aryl alcohol dehydrogenase and (20) a monooxygenase. K = *Kpn*I, E = *Eco*RI.

has been observed in several known DNA binding proteins (e.g. GAL4), supporting the hypothesis that *aflR* is a regulator of AF gene expression (Pan and Coleman, 1990). Unlike *verA*, no hybridization was observed when *A. flavus aflR* was used to probe an *A. nidulans* genomic DNA library. Comparison of the *A. flavus* and *A. parasiticus aflR* sequences with those of other zinc cluster genes allowed us to design a single degenerate primer that was used for sequencing reactions using fragments from the *verA* genomic region. Sequence was obtained from one fragment and further analysis demonstrated that this region likely encodes an *aflR* homolog which we have named *AnaflR* (Brown et al., 1994). *AnaflR* is located between the PKS gene and *verA* in the sterigmatocystin gene cluster (**Figure 4**).

Our work with *verA* indicated that enzymatic genes were conserved in the ST and AF gene clusters. We are also interested in determining if regulatory mechanisms are conserved. Towards this goal, we have asked if *A. flavus aflR* (*AfaflR*) functions in *A. nidulans*. A plasmid containing the *niiA* promoter fused to the *AfaflR* coding sequence was used to transform *A. nidulans* (*niiA*, encoding nitrite reductase, is induced by nitrate). The resulting transformant, TRB76, was then grown in nitrate medium for 2 to 12 hours, a time frame when *A. nidulans* normally doesn't produce ST (Butchko et al., in preparation). The results showed that whereas wildtype *A. nidulans* did not express *verA* or other ST genes at these early time periods, TRB76 did (Brown et al., 1994). These results indicate that the expression of *AfaflR* in *A. nidulans* leads to the expression of *verA* and other known ST pathway genes. These experiments suggest the potential conservation of a regulatory factor involved in mycotoxin production in *A. flavus*, *A. nidulans* and *A. parasiticus*.

(iii) The sterigmatocystin gene cluster. The identification of four ORFs on either side of *verA* suggested that we located a ST gene cluster. Analysis of the DNA sequence of the four ORFs by computer databases revealed that three

of the four ORFs could encode enzymes that had been predicted as necessary for ST biosynthesis. Sequences for ORF1 and ORF2 (e.g. *verB*), both downstream from *verA*, have been reported recently (Keller et al., 1994). These genes encode products which show homologies to the gamma subunit of elongation factor 1 (Keller et al., 1994; Maessen et al., 1987) and a cytochrome p450 monooxygenase (Keller et al. 1994; Yokotani, et al., 1989). ORF3 and ORF4 show identities to an aryl alcohol dehydrogenase and a cyclohexane monooxygenase respectively (Keller et al., unpublished data). In a joint effort with Dr. Tom Leonard's laboratory (Yu and Leonard, 1994), we have also started sequencing the PKS and *anaflR* containing cosmid, pL11C09. Preliminary results have identified 17 ORFs on this cosmid whose transcripts are co-regulated with *verA* (**Figure 4**, Brown et al., in preparation). The majority of these ORFs are predicted to encode activities proposed to be needed in ST biosynthesis (**Figure 1**, Brown et al., 1994, Bhatnagar et al., 1992, Dutton, 1988). This data suggests that there are more than 20 genes involved in ST biosynthesis in an approximately 56 Kb region located on the smallest chromosome of *A. nidulans* (Brown et al., in preparation, Keller et al. 1994).

Acknowledgements. Funding for different projects in T.H.A. and N.P.K.'s programs were provided by USDA-ARS #58-6435-2-129, NRI Competitve Grants Program/USDA award #93-37201-9405 and Eli Lilly and Company, Indianapolis, IN 46285.

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