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Project Summary **and** **Research Report** **Regulation and Inhibition of** **Secondary Metabolism**

Food Science & Human Nutrition



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Table of Contents

Acknowledgements.....3

INTRODUCTION

Fungi as a Model Organism.....4

Secondary metabolism.....7

Aspergillus flavus/parasiticus and Aflatoxin
Aspergillus nidulans and Sterigmatocystin

Economic Importance and Health Significance (Aflatoxin)8

Current Control and Alternative Methods to Aflatoxin Contamination.....11

Research Background.....12

Regulation and Inhibition of Secondary Metabolism.....14

RESEARCH

Research to Date (Initial Research Plan).....15

Research Goals, Questions and Hypothesis.....21

Research Results and Discussion.....25

Experiment I.....25

Experiment II.....27

Experiment III.....34

Future Research.....42

Overall Research Application, Bigger Picture.....45

References.....47

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Fungi as a Model Organism

According to the writings of Horace, the word fungus comes directly from the Latin word *fungus* which is derived from the Greek word *sphongos/σφουγγος* – meaning ‘sponge’ (AbsoluteAstronomy.com). It refers to the macroscopic structures and morphological development of mushroom and molds. For many years, taxonomists believed that fungi are more similar to plants based on their characteristics and similarities in lifestyle. Fungi have appeared to diverge some one billion years ago and while some morphological, biochemical and genetic features are similar to other organisms; others are unique to fungi (Wikidoc.com).



Fig 1.0 – Growth of fungi on common household food, strawberry. Although these fungi might not be toxic, their growth renders the food product as ‘unacceptable’.

In households, a fungus has developed a bad reputation over the years due to its role in food spoilage, growth on damped wood and on bathroom walls. However, due to the versatility and superior competitive abilities, the fungi kingdom has gained its reputation over the years especially in research. Fungi are lower eukaryotes – thus excellent models to study complex eukaryotic processes. When compared to using the animal model, fungi are attractive model systems to study due to its advantage of easy cultivation on simple media and short growth time. These model systems have created an easy access to molecular and classical genetics. Research on the model organism *Neurospora crassa*

began in the early twentieth century and has contributed to providing a system to study cellular differentiation, development and eukaryotic biology (Galagan, Calvo et al. 2003). *Neurospora* is a filamentous fungus that has over 70 years of history providing the basis of the study of molecular and genetic tools.

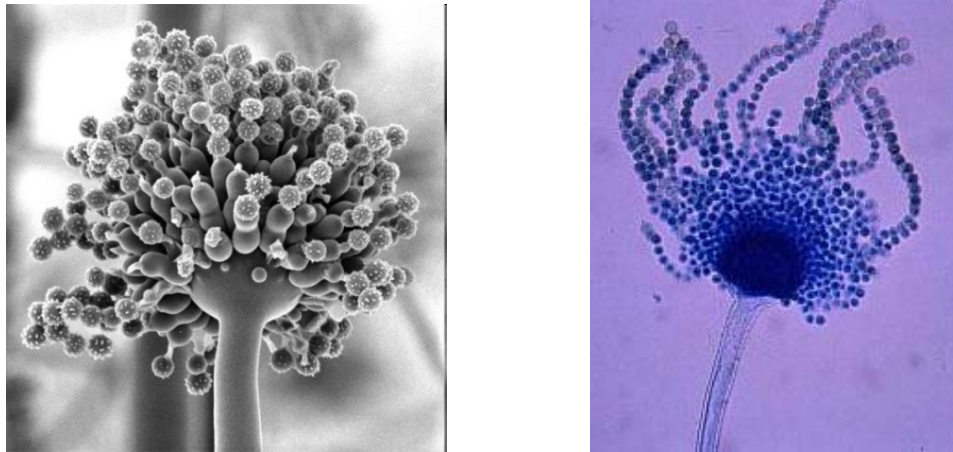


Fig 1.1 – Electron microscopy of *Aspergillus* spp.

Penicillin produced by *Penicillium* is the most famous fungal product and has been used as an antibiotic for many years now in the pharmaceutical industry. Penicillin was discovered by accident by Alexander Fleming and this secondary metabolite has been used as an antibiotic due to its lack of toxicity and efficacy in humans (Drews, uuml et al. 2000). Other industrial usage of non-pathogenic strains includes those in the food industry; *Aspergillus niger* and *Aspergillus oryzae* for the production of high fructose corn syrup and for the fermentation of soy sauce respectively. *Aspergillus oryzae* is especially popular in China and Japan for the production of *koji*, which is a main ingredient for the fermentation of soy sauce, *miso* and *sake* (Barbesgaard, Heldt-Hansen et al. 1992). Enzyme technology has also made use of fungal enzymes – conversion of biomass to bio-fuel and bacterial biofilm removal (Foreman, Brown et al. 2003; Orgaz, Kives et al. 2006). The development and advances of using fungi in food technology and the pharmaceutical industry is good evidence that fungi (or more commonly known as mold) do have valuable purposes.

While some fungi are termed ‘good fungi’, there are another group of fungi which cause deleterious effects due to the production of potent poisons and toxins. Aflatoxins and trichothecenes are group fungal metabolites that are produced by *Aspergillus* spp. and

Fusarium spp. (Yu and Keller 2005). These fungal metabolites are very toxic substances that result in contamination of the food supply (human and animal feed), deleterious health effects on both humans and animals and negative economic impacts as well.

Fungi are usually soil-borne organisms that obtain nutrition from decaying plant and animal material. These lower eukaryotes have superior competitive abilities and are well adapted to the soil. They can live and survive in almost any environmental niche under stress conditions as well. Different toxin producing species are found in different geographic locations depending on temperature and moisture availability (Payne & Yu 2010, book).

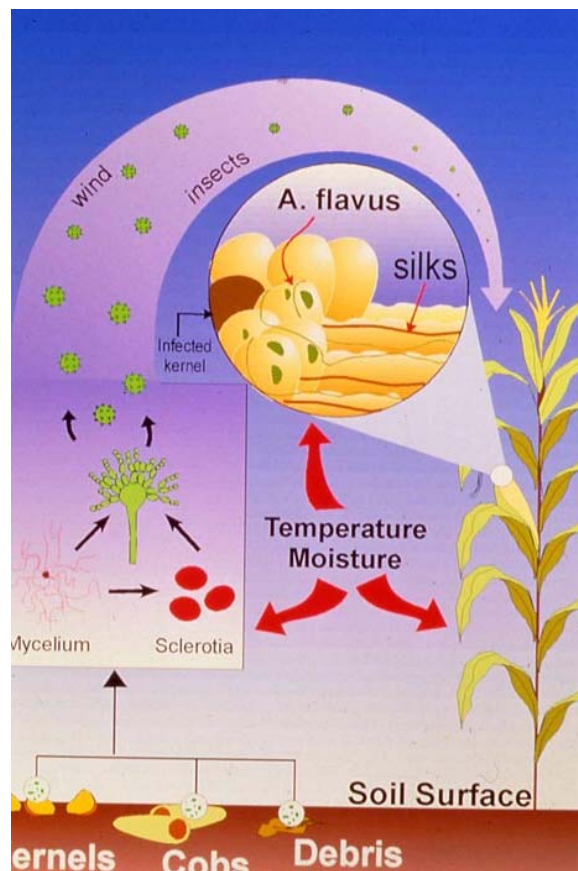


Figure 1.2 – Life cycle of *A. flavus*. The fungus is a soil-borne organism and survives by producing mycelia or sclerotia. Under favorable environmental conditions, mycelia or conidia is transported by wind/insects onto crops where the fungus infects and produces toxins. Sclerotia are survival structures under stressful environmental conditions (Picture obtained from aspergillusflavus.org)

Secondary metabolism

In primary metabolism, compounds are broken down to yield energy for vital processes necessary for the maintenance of life – cell growth, development and reproduction. Secondary metabolism does not affect primary metabolism, in that the producer organisms can still undergo normal growth without synthesizing secondary metabolites. The production of secondary metabolites (products of secondary metabolism), although not deleterious to the organism has been shown to aid in adaptive functions during late stages of growth. Plants and fungi produce a range of these low molecular weight compounds through very unique and complex biochemical pathways, often in response to environmental conditions that trigger their production (Price-Whelan, Dietrich et al. 2006). The study of secondary metabolism and the production of secondary metabolites have contributed significantly to the pharmaceutical and medical industry – this work resulted in the discovery of morphine and quinine (plant metabolites) as well as fungal metabolites such as streptomycin, penicillin and cephalosporin (Martin and Liras 1989; Keller, Turner et al. 2005). However, the mycotoxins another group of secondary metabolites that includes aflatoxin and trichothecenes are potent poisons produced by fungi. The production of secondary metabolites has been of great interest because the manipulation and control of metabolite production can either to increase the production of advantageous metabolites or decrease/block the synthesis of deleterious metabolites.

In terms of research in secondary metabolism using *Aspergillus* as a model organism, research laboratories across the United States and Europe have been studying strains of *A. flavus*, *A. parasiticus* and *A. nidulans*. Research knowledge on understanding the molecular mechanisms that govern the regulation of secondary metabolism has been gained mostly from the study of the aflatoxin gene cluster in *A. flavus* and *A. parasiticus* and the sterigmatocystin gene cluster in *A. nidulans* (Brown, Yu et al. 1996; Yu, Butchko et al. 1996). The complete genome of *A. flavus* and *A. nidulans* has been sequenced (aspergillusflavus.org; broadinstitute.org). Studies done at Michigan State University and all works containing initial research plan has been dealing with *A. parasiticus* as the model organism and studying the regulation of secondary metabolism using the aflatoxin

gene cluster. In this work we rely on the genome of *A. flavus* because a complete genome for *A. parasiticus* is not available. Experiments done using primers designed from *A. flavus* worked with many genes in *A. parasiticus* (Miller, Roze et al. 2005; Hong and Linz 2009). Research work/experiments at BOKU Vienna were done with *A. nidulans* as a model organism and studying the regulation of secondary metabolism using the sterigmatocystin gene cluster. Thus, most of the background knowledge including the introduction and the conclusions will focus primarily on *A. parasiticus* and the aflatoxin gene cluster. However, our end goal is to understand the regulation and inhibition of secondary metabolism in fungi in general.

Aflatoxin: Economic and Health Significance

Aflatoxin is a toxic substance produced by the fungi *A. flavus* and *A. parasiticus*. “A” stands for *Aspergillus* and “fla” for the species *flavus*. Aflatoxin literally means poison from fungus. It is a deleterious group of secondary metabolite and has been found to be extremely potent carcinogens known to cause liver and lung cancer (Fox, Feng et al.; Dvorackova, Stora et al. 1981). One of the structural forms, aflatoxin B₁ is most toxic to humans and animals.

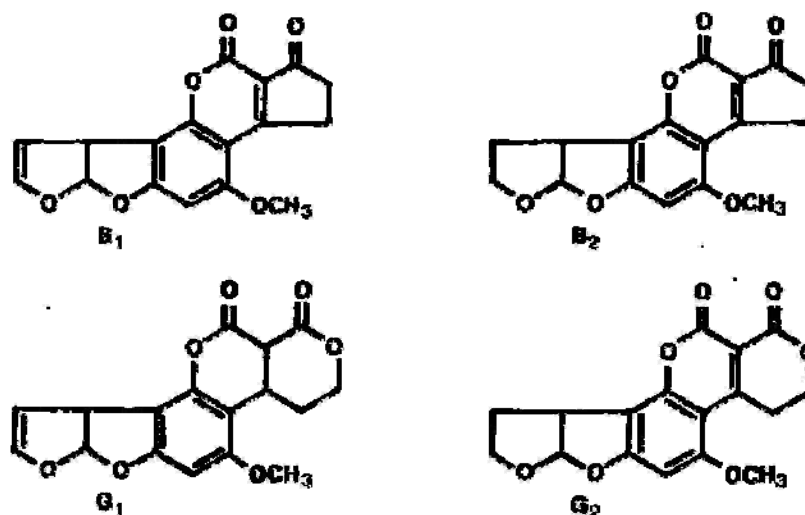


Figure 2.0 – Structures of major groups of aflatoxin

Throughout history, products of secondary metabolism such as the mycotoxins have had a negative impact in terms of economic costs and health significance. The discovery of aflatoxins (toxins produced by fungal species *A. flavus* and *A. parasiticus*) dates back to 1960, when peanut meal that was suppose to be fed to turkeys were contaminated with toxins produced by fungus. More than 100,000 young turkeys died in poultry farms in England with no common medical symptom related to these deaths. Their general clinical appearance suggested that some poisoning had taken place. They were generally dull and lifeless and many of them were in a semi-comatose state and within half an hour more than a dozen others had died. This new disease was called “Turkey X Disease” (Colwell, Ashley et al. 1973). In the United States, the same situation occurred in 1965, when more than 250,000 turkeys went through the same symptoms. These incidences were traced back to aflatoxin contaminated feed given to these turkeys.

In the animal industry, aflatoxin contaminated feed decreases growth rate and decreases the efficiency of the animals to convert feed to body mass. This again is a huge economic loss to the farmers and producers. The discovery of these toxins in animal feed and possibly the human food supply created awareness among the agricultural sector and researchers on the occurrence of contaminations in food and feed in crops. Aflatoxin is primarily produced by species *A. flavus* and also *A. parasiticus*. Under optimal environmental conditions, the growing fungus produces toxins that affect corn, cotton seed, peanuts, treenuts. These agricultural commodities that are contaminated by aflatoxin either enter the food stream or they contribute to the estimated \$500,000,000 annual cost associated with identification and diversion of contaminated crops ((Robens and Cardwell 2003). The United States Department of Agriculture (USDA) set the advisory level for aflatoxin at 20 parts per billion (ppb) on contaminated crops. This causes a huge impact on farmers and producers because contaminated crops are of no value. This advisory level for aflatoxin possess two problems; nationally and internationally. This problem is more of a concern in developing countries because contaminated feed causes inability to sell crops worldwide.



Figure 2.1 – *A. flavus* colonize a corn kernel.

Other than economic costs, aflatoxin is also known to be potent hepatocarcinogens (liver cancer) and has also been associated with lung cancer. Aflatoxicosis is caused by the ability of this compound to randomly mutate DNA specifically at the liver to cause liver tumors (Fox, Feng et al.). This is why most cases of aflatoxicosis have been associated with liver cancer. Developing countries like Africa and South Asia where regulatory measures are less stringent; there are higher incidences of contaminated crops and aflatoxicosis (Williams, Phillips et al. 2004). There have been deaths associated with consumption of contaminated crops – 125 people were killed in Kenya (2004) from consuming aflatoxin-contaminated crop (CDC). Thus, the elimination of aflatoxin is a critical health and economic problem in the United States and throughout many regions of the world.

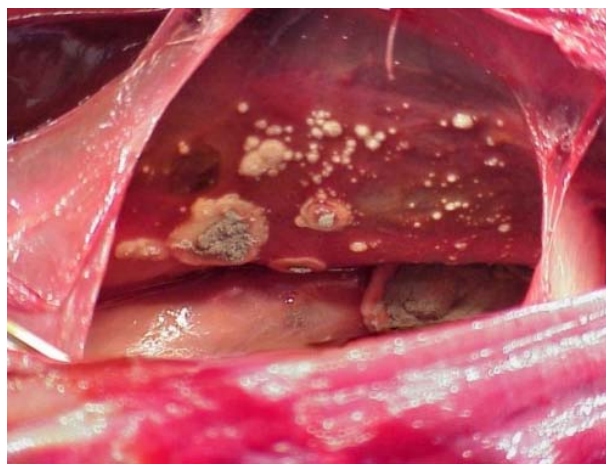
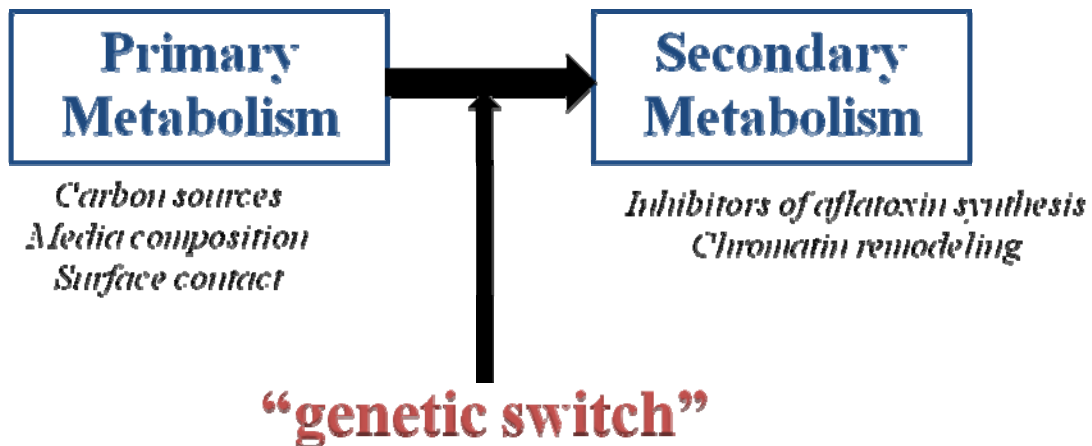


Figure 2.1– Aflatoxicosis infecting the lungs of animals.

Current Control of Aflatoxin Contamination

- (1) Contaminated crops are destroyed through mass burning
- (2) Biocontrol agents
- (3) Decontamination through ammoniation and enzyme processes
- (4) Control conditions to prevent fungal growth/ toxin inducing conditions – cost
- (5) Treat with pesticides and fungicides – other concern with health risk
- (6) Clay/silicate filter for animal feed

Alternative Control of Aflatoxin Contamination



- (1) understanding the role of fungal and plant metabolites in blocking aflatoxin accumulation and gene expression by identifying unique fungal targets and natural product inhibitors to block toxin synthesis on crops (storage or in field)
- (2) characterizing the genetic switch that activates aflatoxin gene expression and to develop a detailed model that predicts the mechanisms by which this switch regulates the timing and level of aflatoxin synthesis

Research Background

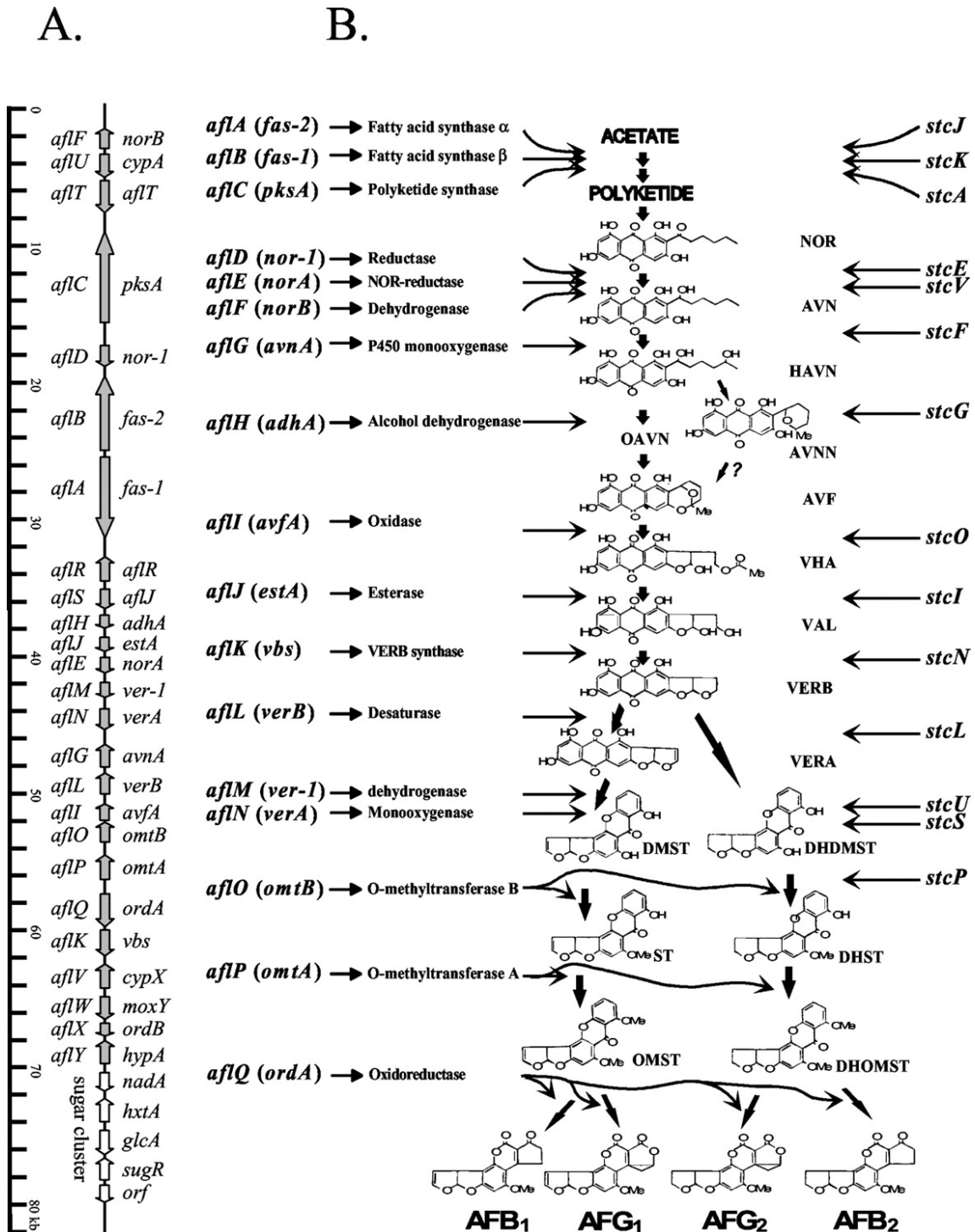


Figure 3 – Aflatoxin biosynthesis. A. Genes involved in aflatoxin biosynthetic pathway. B. Enzymes required for the conversion of intermediates in the pathway.

There are 25 genes responsible for the enzymatic conversions in the aflatoxin pathway. All of the known genes for the pathway are clustered within a 70-kb DNA region. Genes encoding the proteins required for the synthesis of secondary metabolite are found to be clustered in fungal genomes. The synthesis of these products is found to be responsive to environmental cues (carbon, nitrogen, stress, pH and temperature). Environmental stimuli trigger signaling cascades that control expression of both global regulation and AF pathway specific transcription factors.

Regulation and Inhibition

The regulation of secondary metabolism is often a very complicated process in molecular biology because the knowledge on the processes that govern the onset or production of secondary metabolism is very scarce. Also, the link between primary metabolism and secondary metabolism is neither a direct nor a concrete one. However, we hypothesize that primary metabolism provides some metabolic precursors for secondary metabolism, hence factors that affect primary metabolism may also have the ability to regulate and inhibit secondary metabolism. In our work, the regulation of secondary metabolism is important because controlling factors that govern the production of secondary metabolites will help manipulate the outcome – the amount and variety of metabolites produced.

The inhibition or the ability to down-regulate the production of secondary metabolism is essential in controlling processes that produce metabolites that are detrimental to humans and to the environment. The ability to dissect a metabolic pathway of secondary metabolism will enable us to inhibit the production of a harmful metabolite while increasing the production of another possibly by manipulating the precursors of secondary metabolism (end products of primary metabolism).

Regulation

- (1) Nutrient regulation - carbon and nitrogen sources
- (2) Rich media vs. Minimal media
- (3) Surface contact (solid vs. liquid media)

Inhibitors

- (1) Natural fungal metabolites – wortmannin, crotyl alcohol
- (2) Food safe gaseous compounds – ethylene, carbon dioxide, willow bark volatiles
- (3) Target of chromatin remodeling – curcumin, Trichostatin A

Initial Research Plan at BOKU Vienna

Currently, our lab (Michigan State University) is focused on (1) characterizing the genetic switch that activates aflatoxin gene expression and to develop a detailed model that predicts the mechanisms by which this switch regulates the timing and level of aflatoxin synthesis AND **(2) understanding the role of several fungal and plant metabolites in blocking aflatoxin accumulation and gene expression.**

Using *Aspergillus parasiticus* as our model, my research project is directed at finding practical ways to address the problem of aflatoxin contamination of economically important crops using **natural products produced by plants and fungi as inhibitors of toxin synthesis.** Due to the complexity of numerous molecular mechanisms and signal transduction pathways, I have narrowed down my focus on inhibitors to understand the molecular mechanisms of their activity as well as to develop practical methods to effectively use these inhibitors in the field or during crop storage. Much of our effort will be directed at identifying inhibitors that demonstrate promising possibility of usage on the field or during storage of crops. Some inhibitors identified to date are not food safe, however understanding their mode of action is important because this will help identify and confirm specific steps in the aflatoxin biosynthesis pathway that are subject to control.

(B) RESEARCH TO DATE (Progress on the study of aflatoxin inhibitors)

(1) Wortmannin

We have been working with the fungal secondary metabolite, wortmannin isolated from *Fusarium* and *Penicillium*. Wortmannin has been used extensively in the study of mammalian cells as an inhibitor of phosphatidyl inositol (PI)-3 kinase activity (Rondinone, Carvalho et al. 2000; Zhao, Huan et al. 2002). Our lab has shown that wortmannin treatment of *A. parasiticus* grown under aflatoxin-inducing conditions decreases aflatoxin B₁ accumulation. This decrease is accompanied by a large decrease in *ver-1* and *nor-1* (aflatoxin biosynthetic enzymes) promoter activity. We have also shown that exposure to wortmannin increased intracellular cAMP and total protein kinase A (PKA) activity (Lee, Roze et al. 2007). These data prompted us to propose the following model for wortmannin inhibition.

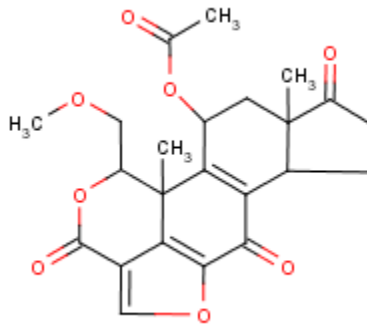
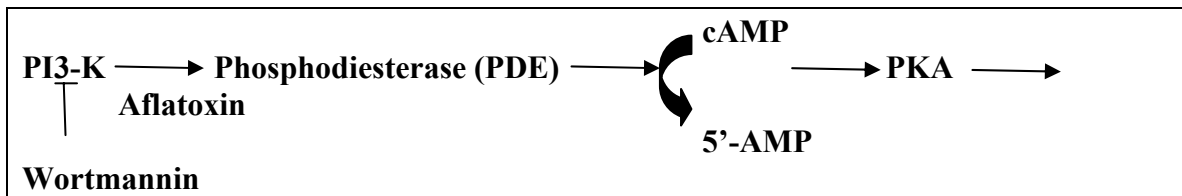


Fig 4.1 - Structure of the fungal isolate, wortmannin.



The inhibition of PI3-K under the influence of wortmannin (a PI3-K inhibitor) is proposed to decrease the activity of PDE; this results in increased cAMP levels, increased PKA activity and decreased aflatoxin production.

(2) Ethylene

A. parasiticus responds to and synthesizes **ethylene**; suggesting that this compound may play a role in the regulating of the life cycle of the fungus. We have shown that ethylene regulates the accumulation and the time of production of aflatoxin on laboratory culture and also on peanuts (Roze, Calvo et al. 2004; Gunterus, Roze et al. 2007). We do not understand the mechanism of ethylene activity at the molecular and genetic level but using ethylene as an inhibitor promises to provide practical means to prevent aflatoxin production on crops because ethylene is food safe, inexpensive and has a long history of use in agriculture (Roze, Calvo et al. 2004). From an agricultural perspective, an ideal inhibitor should be safe, inexpensive and specific in blocking aflatoxin synthesis in the fungus without affecting the development, yield and nutritional value of the crop.

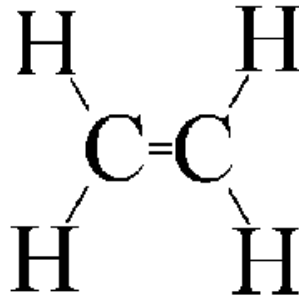


Fig 4.2 – Structure of ethylene.

(3) Aflatoxin gene cluster and chromatin remodeling

2007 shifted our focus to the study of epigenetics to better understand the association between the level of histone H4 acetylation and the activation of aflatoxin gene expression. Our data (Roze, Arthur et al. 2007) suggest that the site of initiation and spread of histone H4 acetylation modulates the timing and order of transcriptional activation based on the arrangement of genes within the aflatoxin cluster. In the case of aflatoxin biosynthetic pathway, alignment in the cluster roughly correlates with ‘early’, ‘middle’ and ‘late’ enzyme activities. I am interested in characterizing the effect of histone acetyltransferase (HAT) inhibitors and histone deacetylase (HDAC) inhibitors on aflatoxin synthesis and how they might interfere with histone H4 acetylation.

(C) Proposed Research

The overall objective of my research at BOKU will be to study the relationship between natural inhibitors and how they function to influence chromatin remodeling.

(1) PI3-K Inhibitor: Wortmannin

We know that wortmannin targets the PI3-K signaling pathway in mammalian cells and that cAMP is one of the key regulators in this pathway. Based on our proposed model of wortmannin inhibition in *A. parasiticus*, when cAMP levels increase, PKA activity also increases and that *nor-1* and *ver-1* are inhibited at the level of transcription. We would like to characterize a possible link between PI3-K signaling pathway to histone modification, and to aflatoxin gene regulation: what this effect of wortmannin on the phosphorylation of CRE1-bp and on HAT activity could be? There are numerous

possibilities to explore the connection between cAMP and CRE1-bp to HAT activity in the presence of wortmannin.

Specific Aim 1. Analyze the effect of wortmannin treatment on CRE1-bp and HAT activity. We propose that wortmannin targets the cAMP/PKA pathway by increasing cAMP levels and increasing PKA activity. We hypothesize that this increase in PKA then prevents the dephosphorylation of CRE1-bp and also prevents the recruitment of HAT; this will stop the initiation of aflatoxin gene expression. From an applied perspective, any compound that is able to block CRE1-bp activation should effectively block aflatoxin synthesis.

(2) HAT/HDAC Inhibitors: Curcumin and Trichostatin A

Curcuma longa rhizome (better known as the spice turmeric), contains a polyphenolic compound called **curcumin** known to possess anticancer activity. (Balasubramanyam, Varier et al. 2004) found that curcumin is a natural and specific inhibitor of p300/CREB-binding protein **HAT activity**. The group also proved that this compound is highly specific to HAT activity and not HDAC activity. We propose to study the effect of this inhibitor on CRE1-bp and HAT activity and on the production of secondary metabolites (aflatoxin).

Another product of secondary metabolism produced by *Streptomyces*, **Trichostatin A (TSA)** is well known as an inhibitor of class I and class II **histone deacetylases (HDAC)** in mammalian cells (Shwab, Bok et al. 2007). Keller's group showed an increase in the production of secondary metabolites in *Alternaria alternata* and *Penicillium expansum* in the presence of TSA. In this paper, they hypothesized that the regulation of chromatin remodeling in secondary metabolism is an efficient way for filamentous fungi to ensure that energetically costly molecules are only synthesized when production is likely to be of advantage to the organism.

One of the unique features of *A. parasiticus* is its ability to produce a secondary metabolite that is not only a potent toxin to humankind but also a toxic substance to the fungus itself. Why does the fungus synthesize a secondary metabolite that is detrimental to itself? From a research standpoint, understanding the epigenetic regulation of secondary metabolism will help us to not only understand the production of metabolites

that are advantage to the organism but also those that may be detrimental to the organism. Using inhibitors at different points of the pathway may provide us with better understanding of how the flow of energy and carbon is channeled to the production of secondary metabolites. From an applied standpoint, we are able to then show that chromatin remodeling in is an effective target for not only inhibition of aflatoxin but as well as the production of secondary metabolites in general.

Specific Aim 2. Investigate the effect of curcumin and TSA on the activity of HAT and HDAC and the correlation to aflatoxin biosynthesis. We hypothesize that applying curcumin under aflatoxin inducing conditions should prevent activation of the aflatoxin gene cluster by preventing the recruitment of HAT. Likewise, TSA should then increase the production of aflatoxin under the same conditions. From a rational standpoint, any compound that blocks the recruitment of HAT should effectively block aflatoxin synthesis.

(3) The role of glucose/sucrose in regulating secondary metabolism

The regulation of extracellular nutrient sensing is an extremely complex network that most likely by-passes the largest family of transmembrane receptor, the G-protein-coupled receptor (Bahn, Xue et al. 2007). Filamentous fungi utilize glucose as the main carbon source, thus they have developed sophisticated mechanisms to sense and transport glucose. There is evidence that in *S. cerevisiae*, the glucose-sensing system works through the cAMP pathway (Bahn, Xue et al. 2007). Although there is a possibility of a presence of a Gpr1 homolog similar to yeast, possible mechanisms for detecting, binding and transport of glucose and sucrose remain to be explored. The amount of aflatoxin produced by the fungus depends on the flow of carbon into the production of acetyl-coA which in turn depends on the amount of glucose/sucrose the organism detects, transports and channels. Thus, understanding exactly how carbon flows into the production of aflatoxin will allow us to manipulate the biosynthesis of this secondary metabolite.

Specific Aim 3. Analyze the effect of varying carbon source under the presence of wortmannin and the effect on chromatin remodeling. When the [glucose] in the

medium is high, GPCR is bound and adenylate cyclase is stimulated, intracellular [cAMP] increases and basal PKA increases. Wortmannin targets PI3-K activity that also affects cAMP/PKA activity. We hypothesize that varying the carbon source under the presence of wortmannin would result in differences in HAT activity and the activation of aflatoxin genes.

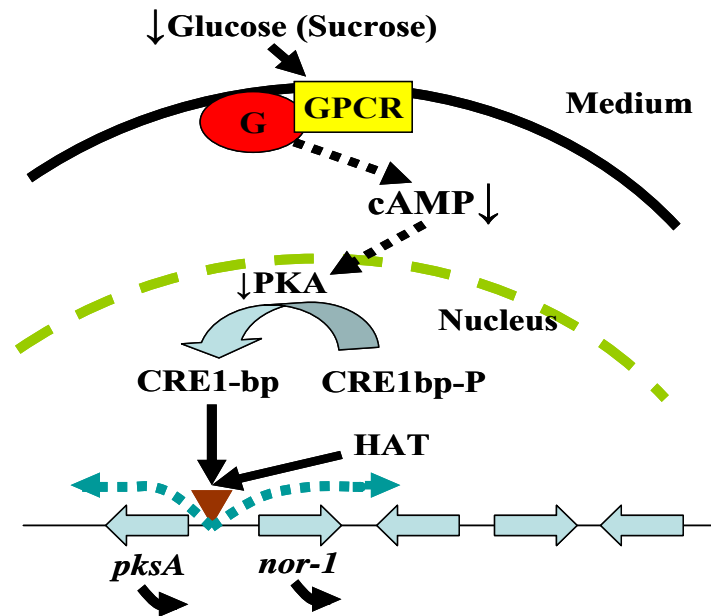


Fig 4.3 – Simplified model to predict the regulation and possible pathway inhibition of aflatoxin biosynthesis. Decreased levels of cAMP/PKA leads to the dephosphorylation of CRE1bp that is bound to 15 CRE1-like sites on the *pksA/nor-1* intergenic region. This causes the recruitment of HAT, and CRE1bp and HAT forms a complex to initiate a bidirectional acetylation of histone H4 – this causes gene activation.

Research Goals, Questions and Hypothesis

Research Aim 1:

To look at the effect of environmental stimuli (nutrient source) in the regulation/inhibition of secondary metabolism by monitoring secondary metabolite production (sterigmatocystin) and gene expression (*aflR*).

Rationale for Research Aim:

(1) Inhibitor Studies

(Refer Research Proposal) Different inhibitory effect on different media with different nutrient source (carbon source – glucose, sucrose); media composition (rich, minimal) and surface contact (solid, liquid).

(2) LIFT Experiments

Transfer growing fungus to new plate with renewable nutrient source – increased norsolorinic accumulation (aflatoxin pathway intermediate) – observable through orange color formation.

(3) Nutrient Studies (data generated by Asjad Basheer)

Preliminary Data

Generated by Asjad Basheer

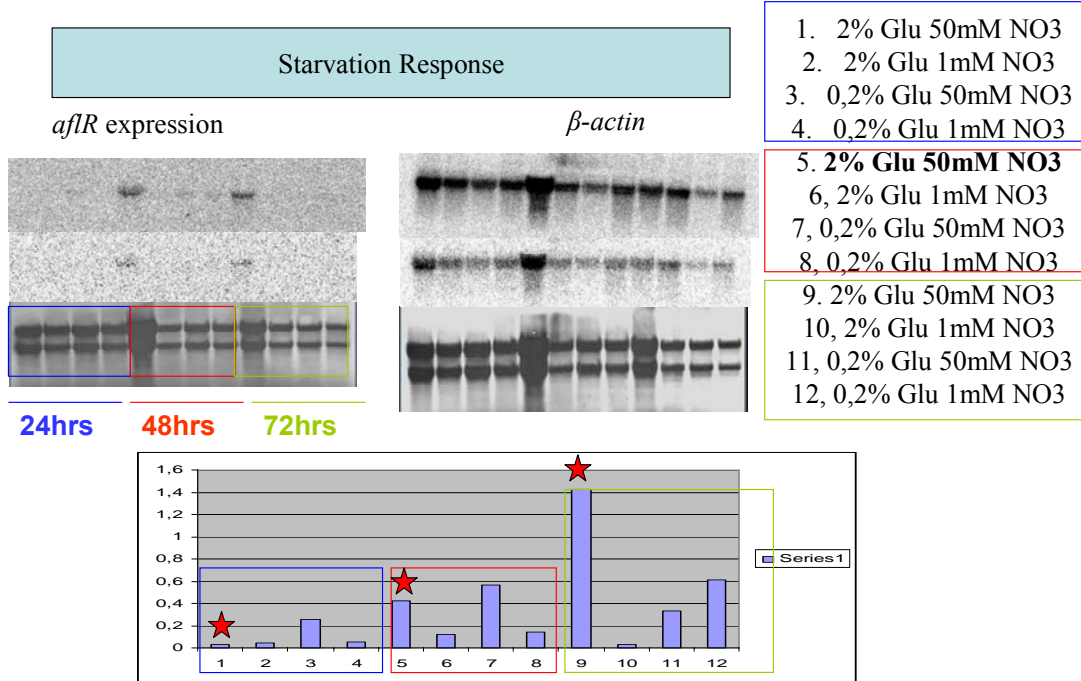
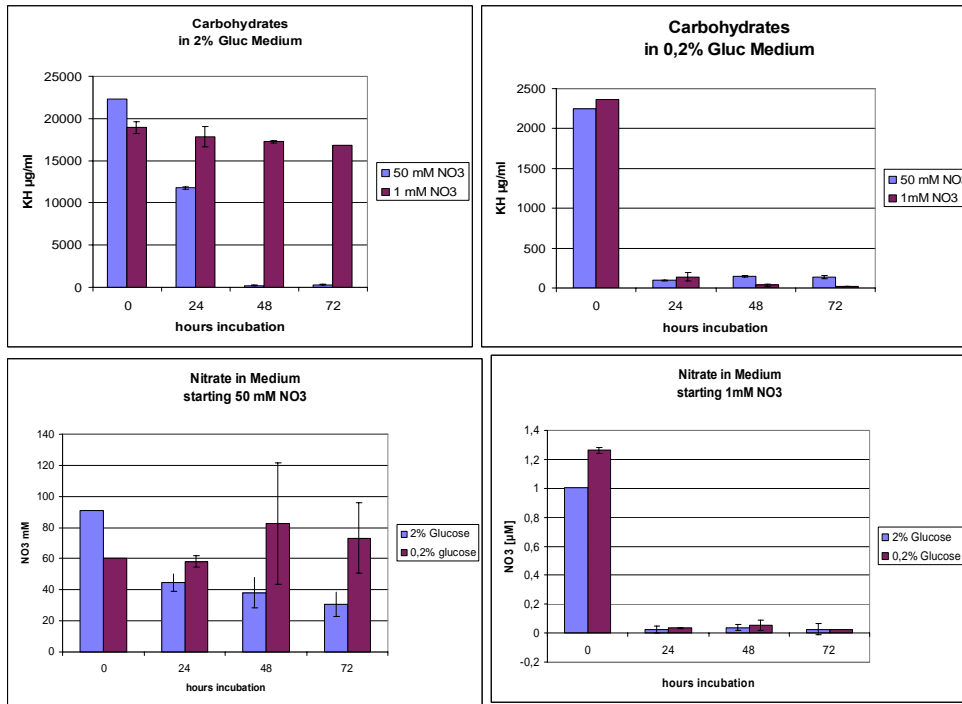


Fig. 5.1 – Nutrient starvation response in different media compositions with data on Northern blot analysis of *aflR* expression.

These data generated by Asjad Basheer and Sandra Böck, a co-worker at Strauss Lab provided some basis for nutrient characterization studies that was used as my experimental basis and media conditions. Just focusing on the trend of the most optimal conditions (2% glucose, 50mM nitrate) *aflR* is expressed according to the typical time point trend whereby at 24hr, very little expression is detected, increasing at 48hr and peaks at 72 hr. However, when different combinations are used (2% glucose, 1mM nitrate – nitrogen starvation response), *aflR* is suppressed at almost all time points. In 0.2% glucose, 50mM nitrate (glucose starvation), *aflR* can be detected as early as 24hr, increases at 48hr but decreases at 72hr. With 0.2% glucose, 1mM nitrate (glucose/nitrogen starvation), trend of *aflR* expression mimics 2% glucose, 50mM nitrate but to a smaller degree. Thus, these observations tell us that glucose/nitrogen starvation response can play a role in regulating, controlling and manipulating *aflR* expression.

Experiment 1 : Glucose 2% / 0,2 %
 NO₃ 50mM / 1mM
 2 biological repetitions

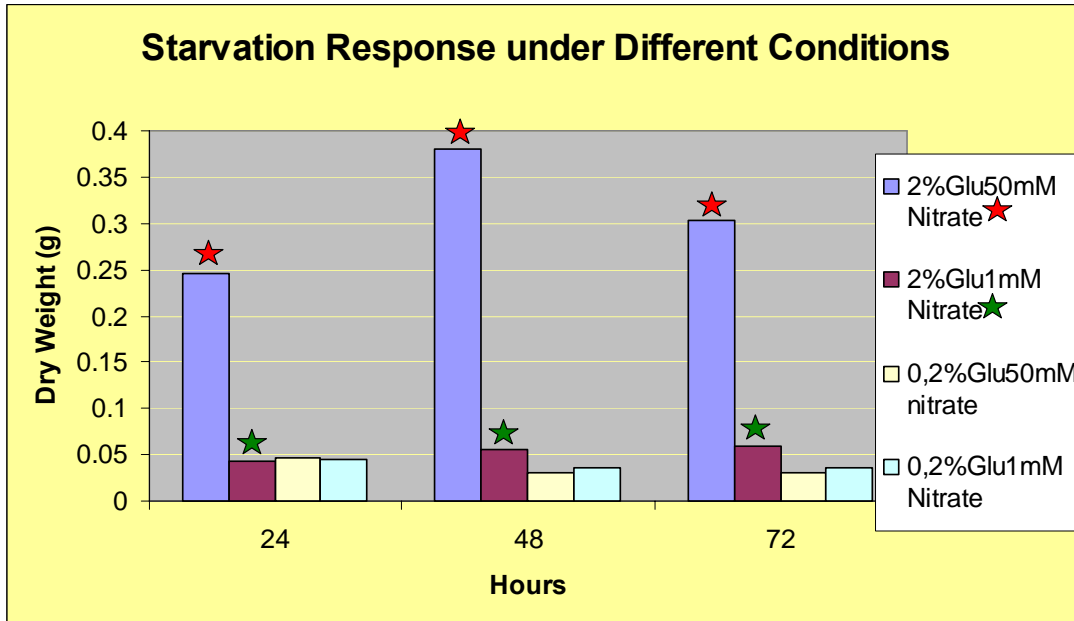


Data generated by Asjad Basheer

Fig 5.2 – These graphs show the metabolism/utilization patterns of glucose/nitrate over time for the media conditions used above.

2% glucose under the presence of abundant nitrate, glucose is used up almost 50% by the first 24hr and by 48hr glucose in the media is almost gone. When glucose is limiting, the percentage of glucose utilization is higher because almost all glucose is used by 24hr for both 50mM nitrate and 1mM nitrate. Does that mean that under nitrogen starvation, nitrate metabolism speeds up? Nitrate concentration (50mM) with sufficient glucose (2%), there is a steady decrease in nitrate utilization. There is no particular trend for data in 0.2% glucose, 50mM nitrate because nitrate seem to be increasing in the media. This is possibly nitrate production *in vivo*. Nitrate starvation under conditions with sufficient glucose showed that almost all nitrate is used up by 24hr. Under glucose/nitrate starvation also showed that all nitrate is utilized by 24hr. Thus, when one source is limiting, the other nutrient is almost utilized completely by 24hr. These data also tell us that there is a correlation between glucose depletion and *afIR* expression.

Biomass



Data generated by Asjad Basheer

Fig 5.3 – This graph provides biomass data for all conditions listed above.

Biomass data was interesting because when comparing 2% glucose conditions, excess nitrate (50mM) in the media caused approximately 5-8 fold more mycelia mass than when nitrate was reduced to 1mM. Figures 5.1, 5.2 and 5.3 establish that - preliminary data generated from my studies used the basis of results obtained from nutrient conditions in these series of experiments: glucose minimal media (GMM) with 2% glucose, 50mM nitrate to ensure *aflR* expression.

Hypothesis:

Questions that I am trying to answer with these studies/what I am trying to accomplish and how worthwhile are they to pursue?

“Nutrient regulation has a greater effect on metabolic pools of precursors than direct effect on pathway gene expression”

Research Results and Discussion

Experiment PART 1

From the rationale of the LIFT experiments, we wanted to observe the effect of the addition of a carbon source at different time points and its effect on transcript expression levels, *aflR* and accumulation of sterigmatocystin. The question that I wanted to ask in this experiment is whether it is possible to manipulate the timing and production of sterigmatocystin by the addition of carbon source.

First experiment –

- (1) Real time PCR to quantitate the expression of *aflR*
- (2) Northern blot analysis to support real time data

Observation – ‘interesting’ real time data but Northern blot showed no *aflR* expression (control gene β -actin was fine).

Second experiment – to repeat the first

- (1) Northern blot analysis
- (2) TLC – metabolite accumulation

Observation – again on Northern blot showed no *aflR* expression

TLC – also showed no ST production

Third experiment (Time course experiment 24, 48, 72 hr)

Observation - Northern blot analysis showed no *aflR* expression again

TLC showed no ST production

Discussion (Why experiments did not work?)

I did not detect *aflR* expression in Northern blot analysis and non-detectable levels of accumulation of sterigmatocystin because I was inoculating the wrong amount of spores – calculation error, I inoculated 20x less spores than what I was suppose to – causing *aflR* not to be detected at 24h, 48h and even 72h. Population density in liquid culture is very important for signal of secondary metabolism/trigger *aflR* expression. On the other hand, when more spores are inoculated (20x more than the actual amount), *aflR* expression can be detected as early as 24hr. This effect for the signal of secondary metabolite production may be an effect of quorum sensing and population density.

Other explanation, when there are less spores in the medium, glucose is utilized to a much slower extend compared to when there are more spores in the media. So, when spores remain at a lower count, glucose repression is always going on, so secondary metabolism is not initiated. This is the opposite for when there is sufficient mass of mycelia in the media. Thus, this transition phase from active growth to stationary phase happens slower in less dense culture compared to cultures with higher density.

Experiment PART 2

Solid MM WT ST



To obtain preliminary data on the effect of glucose, nitrogen and ammonium on solid media, solid minimal media was used as a growth medium with 1% glucose and 10mM nitrogen/ammonium. Additional glucose was added to samples (++G1 and ++G2) to observe the effect of an additional carbon source. Sample descriptions are as follows:

++G1/++G2 1% glucose minimal media + additional 1% glucose + 10mM nitrate

NO1/NO2 1% glucose minimal media + 10mM nitrate

NH1/NH2 1% glucose minimal media + 10mM ammonium

Aspergillus nidulans WT strain was grown freshly on minimal media and a spore solution was made. 10 μ L of 10⁸ spores/mL spore solution was used to center inoculate solid media. Plates were left to grow for 5 days at 37°C. Sterigmatocystin was extracted using organic extraction and separated by thin layer chromatography.

Organic extraction of ST in solid media: Briefly, the cap of a 50mL conical tube was used to cut out the core of the plate. This core was grinded with 10mL acetone (50% v/v) until a homogenized mixture is formed. This mixture was vortexed for 30 secs and 5mL of chloroform was added. This mixture was left overnight at 4°C for separation. 4mL of the chloroform layer was transferred to a new tube and evaporated using a vacuum speed centrifuge. The residual compounds were dissolved in 40 μ L chloroform and 15 μ L was spotted and analyzed with a TLC plate (no. 4410221; Whatman, Brentford, UK).

Metabolites were separated using a toluene: ethyl-acetate:acetic acid (80:10:10) solvent system. After separation, TLC plate was left to air dry and then sprayed with 15% aluminium chloride solution (15% w/v in 95% Ethanol). After spraying, the plate was left to dry again and then baked in the oven 80°C for 30 minutes. Sterigmatocystin can be visualized on the TLC plate under UV light.

From solid minimal media experiments, it was observed that:

(1) The addition of glucose (final concentration of 2% glucose/10mM nitrate) reduced the total amount of sterigmatocystin produced when compared to sample with 1% glucose/10mM nitrate.

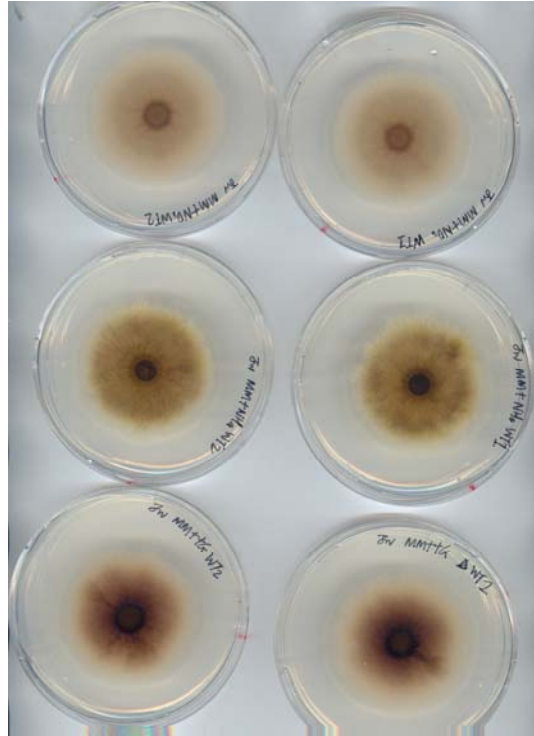


Fig 6.1 – *A. nidulans* WT strain on minimal media

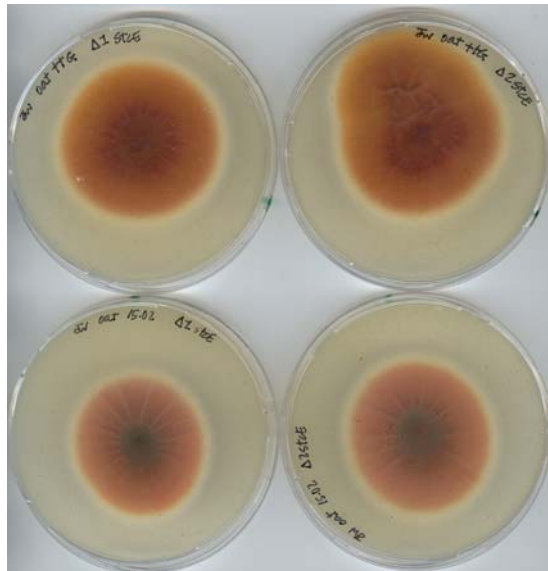
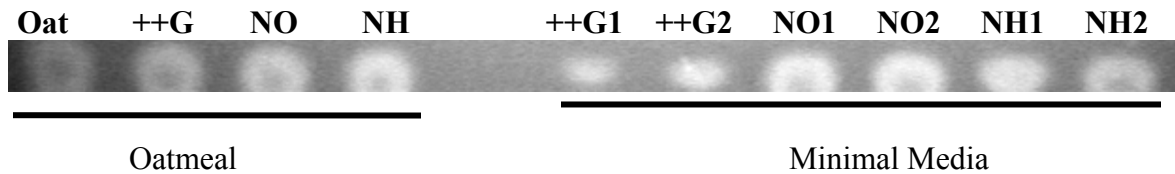


Fig 6.2 – *A. nidulans* $\Delta stcE$ strain accumulates pathway intermediate norsolorinic acid on oatmeal agar, observable by formation of orange color.

Solid Δ stcE NOR



To obtain preliminary data on the effect of nutrients in complex/rich media (oatmeal) vs a minimal media, an *Aspergillus nidulans* Δ stcE strain was used. This strain produces an intermediate metabolite called norsolorinic acid that is a visible orange colored substance when grown on oatmeal agar. The effect of glucose, nitrogen and ammonium on rich and minimal solid media was tested. Oatmeal agar and minimal media was used as a growth medium. Additional glucose was added to samples (++G1 and ++G2) to observe the effect of an additional carbon source. Sample descriptions are as follows:

Oatmeal

- Oat Oatmeal agar
- ++G Oatmeal agar + 10mM nitrate + additional 1% glucose
- NO Oatmeal agar + 10mM nitrate
- NH Oatmeal agar + 10mM ammonium

Minimal Media

- ++G1/++G2 1% glucose minimal media + additional 1% glucose + 10mM nitrate
- NO1/NO2 1% glucose minimal media + 10mM nitrate
- NH1/NH2 1% glucose minimal media + 10mM ammonium

Aspergillus nidulans Δ stcE strain was grown freshly on minimal media and a spore solution was made. 10 μ L of 10⁸ spores/mL spore solution was used to center inoculate on appropriate solid media. Plates were left to grow for 5 days. Norsolorinic acid (NOR) was extracted using organic extraction and separated by thin layer chromatography. Organic extraction of NOR on solid media: The cap of small falcon tube (15mL) was used to cut out the core of the plate. This core was grinded in 5mL acetone (50% v/v) until homogenize. The mixture was transferred to a 15mL falcon tube and 2.5mL chloroform was added. Mixture was vortex 30secs and left to separate overnight at 4°C. 2mL of the chloroform layer was transferred to a new tube and evaporated using a vacuum speed centrifuge. Residue was dissolved in 40 μ L of chloroform and 10 μ L was

spotted onto a TLC plate for oatmeal samples and 15µL was spotted for minimal media samples. Whatman #4410221 was used as TLC plates using a solvent system toluene: ethyl-acetate:acetic acid (80:10:10). After running the plate through the solvent system, plate was left to dry and norsolorinic acid can be visualized under UV light.

From rich vs. minimal solid media (norsolorinic acid analysis), it is observed that:

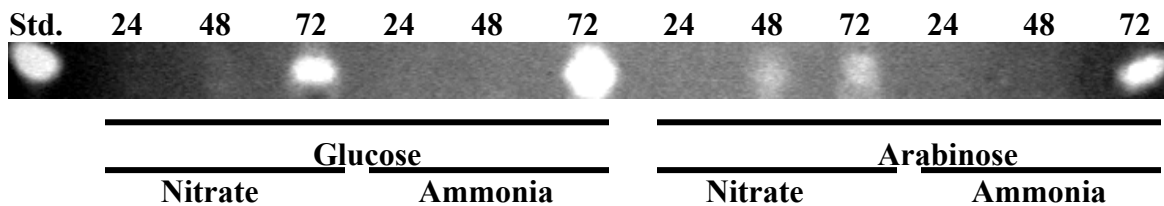
Oatmeal (rich media)

- (1) Comparison of oat vs. ++G The addition of 1% glucose and 10mM nitrate to the rich media increased the production of NOR.
- (2) Comparison of ++G vs. NO These two samples have the same nitrate concentration (10mM) but the addition of 1% glucose decreased the amount of NOR produced when compared to sample with just 10mM NO.
- (3) Comparison of NO vs. NH In rich media, ammonium is possibly a better nitrogen source for accumulation of NOR compared to nitrate.

Minimal media

- (1) Comparison ++G vs. NO Addition of glucose (2% final glucose concentration) decreased accumulation of NOR.
- (2) Comparison NO vs. NH In minimal media, nitrate is possibly a better nitrogen source for accumulation of NOR compared to ammonium.

Liquid MM WT ST



To obtain preliminary data on the effect of an easily metabolized carbon source and a more complex carbon source (arabinose is metabolized through the pentose phosphate pathway) together with a nitrogen source (nitrate or ammonia).

Liquid minimal media was used as the growth medium with 2% glucose or arabinose (v/v) and 50mM nitrate or ammonia. *Aspergillus nidulans* wild type strain (WT) was grown freshly on minimal media (4 days) and a spore solution was made. The final

concentration that was used to inoculate liquid minimal media was 10^6 spores/mL. For the purpose of obtaining an overall idea of how much metabolites are produced at three time points (24, 48, 72 hours), sterigmatocystin was extracted from liquid media by organic extraction and separation through thin layer chromatography (TLC).

Organic extraction of sterigmatocystin in liquid media: Briefly, 25mL growth culture at each time point was collected and 25mL acetone was added. Mixture was placed on an orbital shaker for 3 ½ hours. 20mL of the mixture (1:1 acetone: media) was taken out and 20mL chloroform was added. Mixture (acetone, media, chloroform) was vortex for 30 seconds. Mixture was left to separate overnight at 4°C, then 20mL was evaporated with a vacuum speed centrifuge at room temperature. Sterigmatocystin was redissolved in 40µL chloroform and 20µL of each sample was loaded on a TLC plate (no. 4410221; Whatman, Brentford, UK). Metabolites were separated using a toluene: ethyl-acetate:acetic acid (80:10:10) solvent system. After separation, TLC plate was left to air dry and then sprayed with 15% aluminium chloride solution (15% w/v in 95% Ethanol). After spraying, the plate was left to dry again and then baked in the oven 80°C for 30 minutes. Sterigmatocystin can be visualized on the TLC plate under UV light.

Observations from liquid experiments:

(1) Morphology of mycelial mass

Individual mycelial were formed like balls with some spikes on them and was completely white in color when grown on 2% glucose but when they are grown on 1% glucose they are green and look like a mesh and mycelia mat.

(2) Mycelial mass

Mycelial mass was observably more in conditions of glucose than on arabinose.

(3) Media color

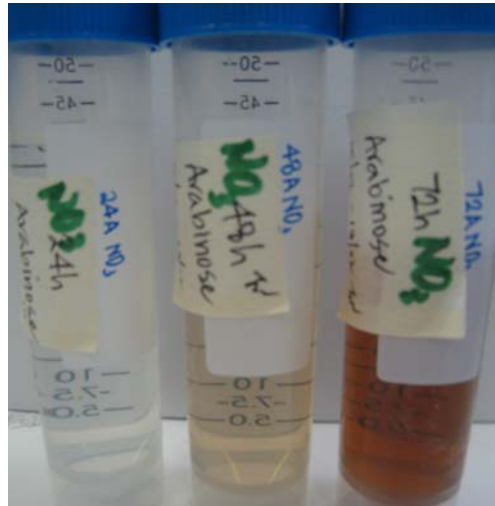


Fig 6.3 – Observing media color change over time in culture conditions with 2% arabinose/50mM nitrate. This color change suggests the possibility of the production of a secondary metabolite pigment. Samples from left to right: 24hr, 48hr, 72hr.

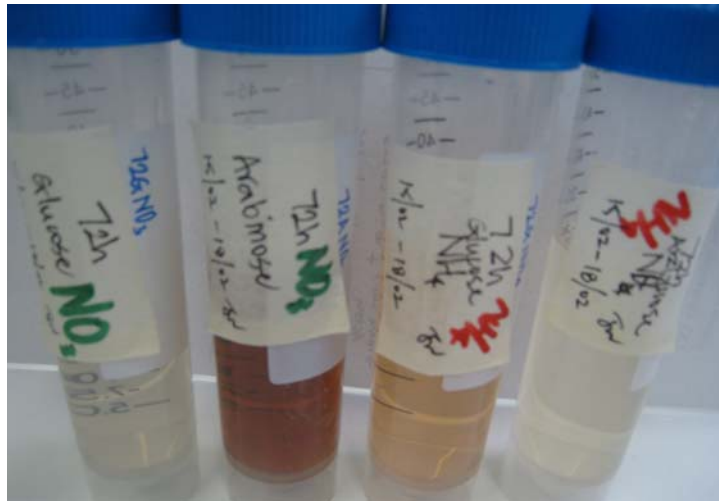


Fig 6.4 – Observation of media color change at one time point 72hr. Samples from left to right, glucose-nitrate, arabinose-nitrate, glucose-ammonium and arabinose-ammonium. Pigment accumulation is the highest in sample with arabinose-nitrate conditions.

From liquid minimal media experiments, it is observed that:

- (1) Comparing all samples at one time point (72 hours) shows that sterigmatocystin is made the most under glucose-ammonium, followed by glucose-nitrate, arabinose-ammonium and the least by arabinose-nitrate.
- (2) Under arabinose-nitrate conditions, sterigmatocystin is presumably observed at an earlier time point compared to the other conditions.

Glucose is a better source than arabinose for ST production; ammonia is a better source than nitrate for ST production with 2% glucose. It was observed before that nitrate stimulates ST production and ammonia represses nitrate production (Feng and Leonard 1998). But what I have observed in these experiments with 2% glucose suggests likewise. Under 2% glucose, ammonia stimulates ST production and nitrate represses ST accumulation.

The expression of major (global) and minor (pathway-specific) regulatory genes that are involved in secondary metabolism in *Aspergillus parasiticus* has been shown to be regulated by the nitrogen regulatory circuit (Chang, Yu et al. 2000). Studies done observing different effects of nitrogen source showed that regulation of nitrate caused a decrease in aflatoxin biosynthesis (Kachholz and Demain 1983). On the other hand, nitrogen source like ammonium sulfate stimulated production of aflatoxin (Shih and Marth 1974).

In *A. nidulans* however, in 1% glucose minimal salts media the nitrogen effect is different. Ammonium repressed sterigmatocystin production, where as nitrate stimulated its production (Feng and Leonard 1998).

Using organic extraction and TLC to observe the production of sterigmatocystin does not provide conclusive evidence on the actual mechanism of production and regulation of this metabolite. Further analysis on transcript accumulation at the RNA level and Western blots for protein analysis need to be conducted to dissect molecular mechanism leading to these observations.

Experiment Part 3

The aim of Experiment 3 was to create an *A. nidulans* strain carrying a reporter gene under the control of secondary metabolism gene promoters (*aflR*, *laeA*) to look at different environmental conditions (carbon, nitrogen etc.). This strain carrying a colorimetric reporter would be an easy screening method to narrow down conditions that trigger gene expression.

AflR is a positive pathway regulator of secondary metabolism. The disruption of *aflR* prevents accumulation of transcripts necessary for aflatoxin biosynthesis; the introduction of an additional copy of *aflR* causes overproduction of aflatoxin. AflR is a zinc binuclear cluster protein found in fungi and is required for transcriptional activation of most structural genes involved in aflatoxin biosynthesis. The promoter region of most aflatoxin genes contains a palindromic sequence of 11bp that AflR protein recognizes and binds to cause the transcription of genes. Recent studies have shown that *aflR* transcription is responsive to a G-protein signaling cascade mediated by protein kinase A. This signaling pathway may mediate some environmental effects on aflatoxin biosynthesis. The sequence of *aflR* in *A. flavus* is different from *A. nidulans*.

LaeA is a global regulatory gene that regulates secondary metabolite production (*laeA* is negatively regulated by *aflR* by a feedback mechanism). LaeA is an *Aspergillus* methyltransferase. *laeA* encodes a protein containing an S-adenosylmethionine (SAM) binding motif. The exact mechanism of *laeA* in regulating secondary metabolism gene expression is still not well understood. LaeA regulates only those genes that are within the secondary metabolite cluster. The hypothesis is that *laeA* alters chromatin structure through methylation (chromatin modification), altering the chromatin structure from silenced heterochromatin to expressed euchromatin, exposing DNA binding sites to make them accessible to transcription.

The rationale of using two promoter regions of a pathway specific regulator, *aflR* and a global regulator, *laeA* would be a good model to observe the effect of these two regulators on secondary metabolism gene expression.

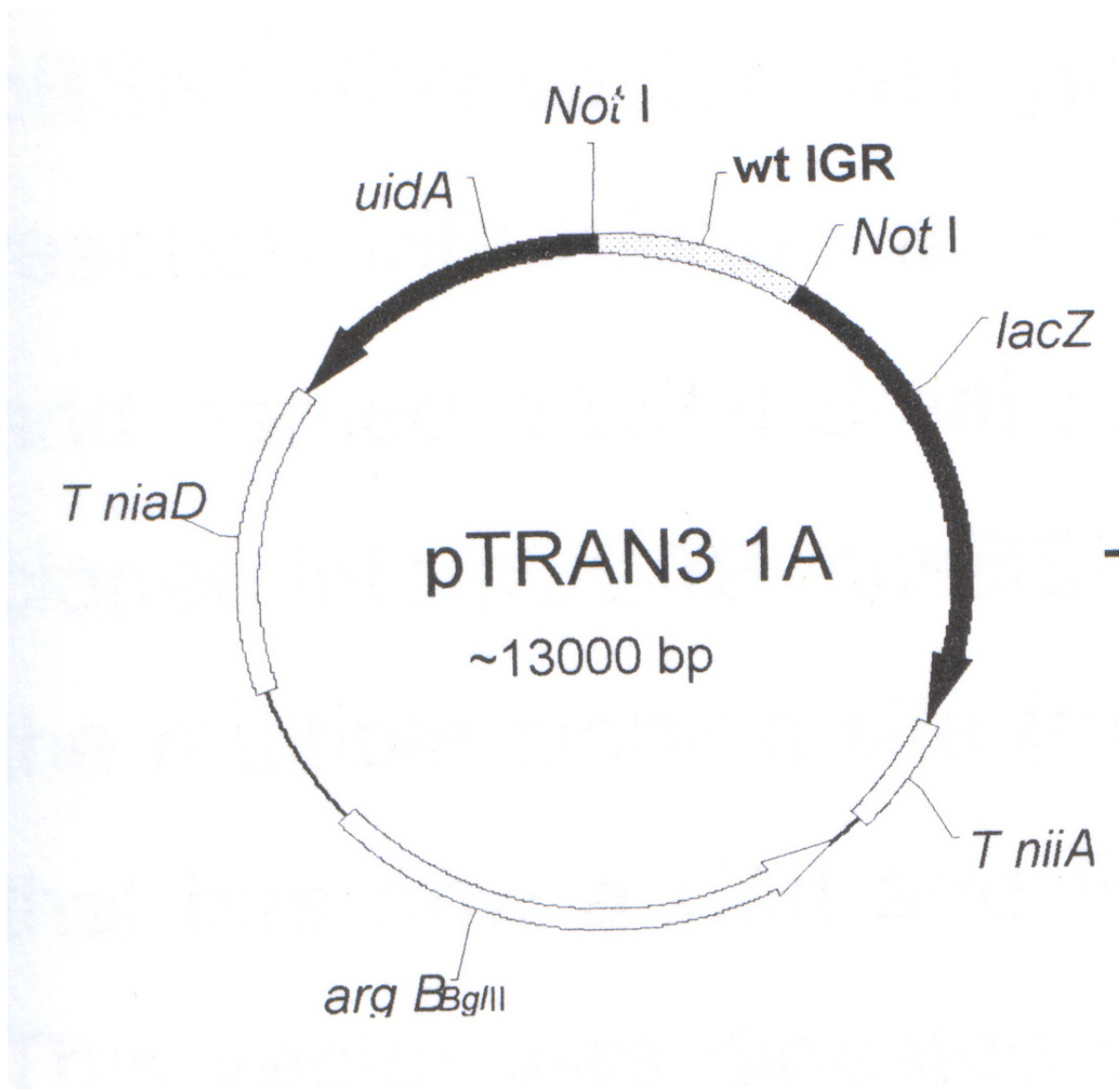


Figure 6.5 – pTRAN3 1A vector that was generated by Robert Pachlinger which was used to clone the promoter region of *afIR* and *laeA* under the *lacZ* expression system. Vector containing intergenic region (*niiA*, *niaD* promoter) with ampicillin resistance.

The forward primer was designed 500bp upstream of the start site of the gene of interest and the reverse primer just before the start site of the gene.

Primers used for this experiment

- (1) *afIR* Not1 prom F
GGGAAAGCGGCCGCTCTTCCGGGACTGGAAGCAGGAC

aflR Not1 prom R

GGGAAAGCGGCCGCGATATTTGCATATGATACAGGC

(2) laeA Not1 prom F

GGGAAAGCGGCCGCCCTAGCCTTCCTAGCCATCC

laeA Not1 prom R

GGGAAAGCGGCCGCGGCGGGGAGACGAGTTCCCACC

(3) lacZ R pTRAN3 1A TATTGGCTTCATCCACCACA

(4) uidA F pTRAN3 1A TCGAGCATCTCTTCAGCGTA

Experimental Report/Summary

(1) Plasmid was taken out of the plasmid collection, 1:50 dilution was made (1 μ L stock + 49 μ L water). 30 μ L competent cells (Invitrogen DH5 α TM Competent Cells Cat. No. 18258-012) was added to 1 μ L of 1:50 plasmid dilution. Reaction tube was placed on ice (4°C) for 15mins. Then, cells were heat shocked for 42°C for 30 seconds. Cells were chilled for 1 min on ice. 400 μ L of pre-warmed (37°C) Luria Bertani (LB) broth was added to the cells. Reaction tube was placed on shaker at 180rpm at 37°C for an hour. Cells were plated on LB ampicillin media overnight at 37°C. Colonies were picked and an overnight liquid culture was made in LB ampicillin (180rpm at 37°C). 4mL of liquid culture was spun down and a QIAprep Spin Miniprep Kit was used to purify the plasmid.

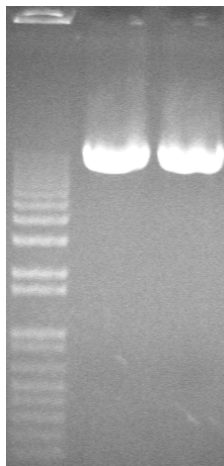


Fig 6.6 – Purified pTRAN3 1A plasmid

A small scale enzymatic digestion was done to confirm restriction sites in the pTRAN3 1A vector. All enzymes were obtained from Fermentas.

	Positive (HindIII)	NotI (actual enzyme)	Negative (PstI)
Plasmid prep.	2µL	2µL	2µL
Buffer	1µL (red)	1µL (orange)	1µL (red)
Enzyme	1µL	1µL	1µL
Water	7µL	7µL	7µL

Expected fragments

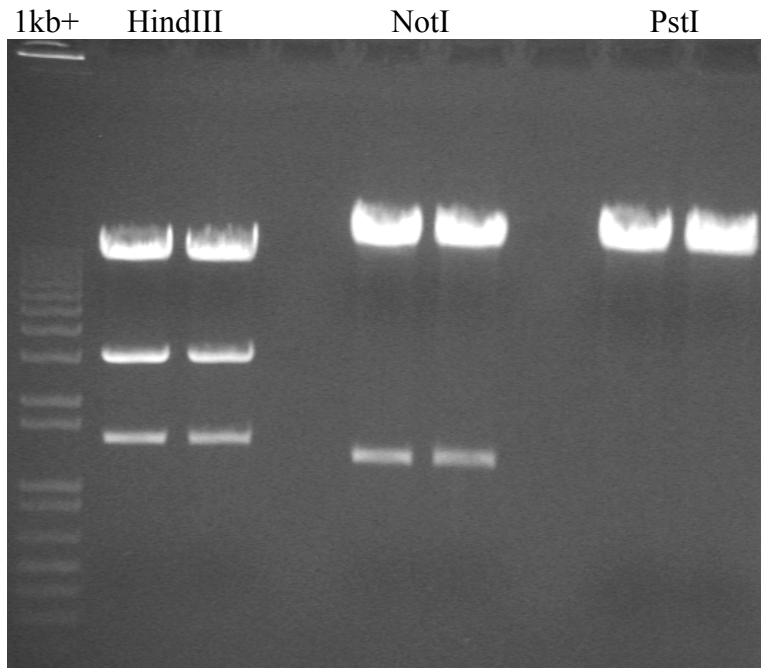
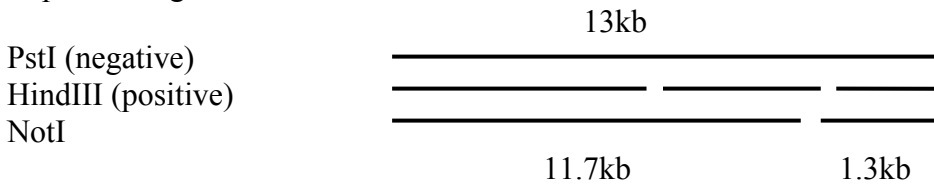


Figure 6.7 - Gel of small digestion on expected fragments. Enzymatic digestion worked as expected.

Large enzymatic digestion was set up with NotI at 37°C overnight.

Plasmid prep	15µL	
Buffer	2µL	
Enzyme	1µL	
Water	2µL	Σ20µL reaction

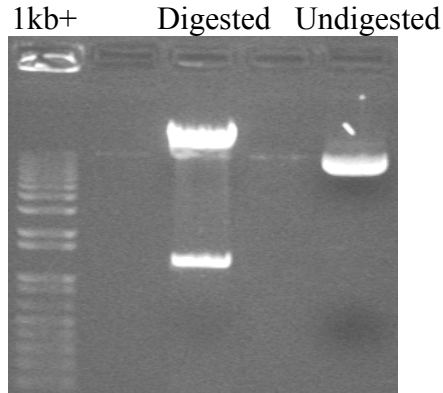


Figure 6.8 – After the overnight large digestion, ran a check gel parallel with the undigested plasmid and purified the large fragment (linearized vector) through MinElute Gel Purification System.

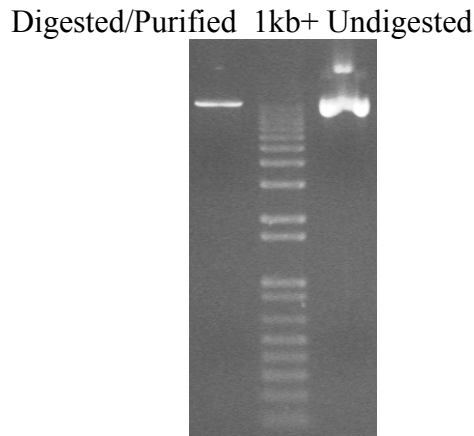


Figure 6.9 – After MinElute Gel Purification.

(2) Promoter region fragments

Expected PCR fragment based on primers:

aflR NotI promoter fragment for insert into pTRAN3 1A vector – 597bp

laeA NotI promoter for insert into pTRAN3 1A vector – 783bp

PCR fragments were generated using Phusion PCR (Finnzymes Cat. No. F-553).

	<i>aflR</i> Not1 prom	<i>laeA</i> Not1 prom	Negative control
Water	17µL	17µL	20µL
PCR mix (2x)	25 µL	25 µL	25 µL
Forward primer (10µM)	2.5 µL	2.5 µL	2.5 µL
Reverse primer (10µM)	2.5 µL	2.5 µL	2.5 µL
DNA Template	3 µL	3 µL	-

Reaction Parameter:

98°C 10s
 98°C 1s
 90°C 5s
 72°C 12s
 72°C 60s
 12°C ∞

} 30 cycles

Phusion fragment was purified using MinElute Column Purification protocol, then a check gel was ran.

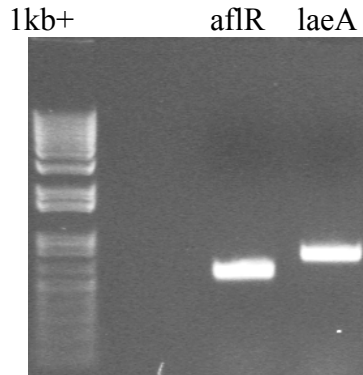


Figure 6.10– Column purified Phusion PCR fragments of *afIR* and *laeA* promoter region both 597bp and 783bp respectively.

NotI overnight digestion was done on both samples (30µL purified PCR fragment, 5µL buffer, 2µL enzyme, 3µL water) at 37°C. After the overnight digestion, an enzyme clean up was done (Qiagen MinElute Reaction Cleanup Kit). Check gel looked similar as Figure – after enzyme clean up.

(2) Ligation Reaction of *afIR* NotI promoter and *laeA* NotI promoter into pTRAN31A

	<i>afIR</i>	<i>laeA</i>	Negative control
pTRAN3 1A purified	1 µL	1 µL	-
Phusion fragment purified	5 µL	5 µL	6 µL
Water	9 µL	9 µL	9 µL

Ligation Reaction – Reaction tubes contained mix as above. Tubes were incubated at 55°C for 10 minutes, then chilled for 1 min on ice. 1µL T4 ligase (Invitrogen Cat. No. 15224-041) and 4 µL 5x buffer for a total reaction of 20µL. Tubes were incubated at 24°C for one and half hours and kept overnight at 4°C.

50µL competent cells (Invitrogen DH5α™ Competent Cells Cat. No. 18258-012) were added to 2µL ligated product (*afIR* and *laeA*) and reaction tube was left on ice for 45

mins. Cells were heat shocked at 42°C for 1 min and tubes were placed back on ice for 2 mins. 1mL pre-warmed LB media was added to the cells and reaction tubes were placed on a shaker at 180rpm at 37°C for one hour. Cells were spun down at 13,000rpm for 1 min and supernatant was decanted. Cells were resuspended and mix in liquid that is leftover in the tube (approx. 100µL) and plated on LB ampicillin media. Plates were left overnight at 37°C.

Twenty colonies were screened for each ligation reaction. Primers that were used for screening were (*aflR* NotI prom F/*lacZ* R and *laeA* NotI prom R/*lacZ* R). Colony PCR was performed on *E. coli* colonies. PCR Reaction was done using REDTaq® ReadyMix™ PCR Reaction Mix – 12.5µL reaction mix, 1.5µL forward primer, 1.5µL reverse primer and 9.5 µL water) for a total reaction volume of 25µL. Toothpicks were used to touch single isolated colonies, and released into PCR reaction tubes with mix.

PCR reaction parameters –

98°C 5min
 95°C 45s } 30 cycles
 60°C 45s }
 72°C 48s }
 72°C 5min
 12°C ∞

Expected size of PCR fragments

- | | | |
|-----|--|--------|
| (1) | <i>aflR</i> NotI prom (597bp) / <i>lacZ</i> R (1243bp) | 1840bp |
| (2) | <i>laeA</i> NotI prom (783bp) / <i>lacZ</i> R (1243bp) | 2026bp |

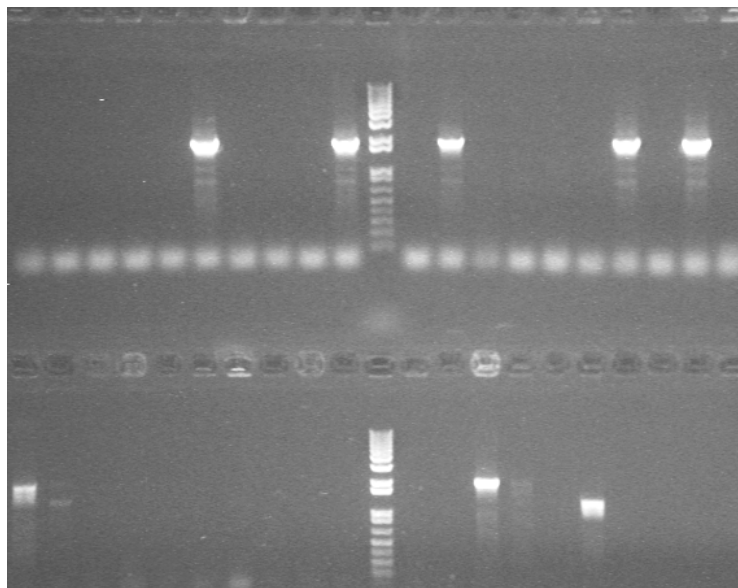


Figure 6.11– Colony PCR reactions of *E. coli* transformation. Five products of the right size were obtained for aflR NotI prom/lacZ and two for laeA NotI prom/lacZ. An overnight culture of colonies that were the right size products were made, and a QIAprep Spin Miniprep Kit (Qiagen Cat. No. 27106) purification was done. Purified plasmids were checked on a gel.

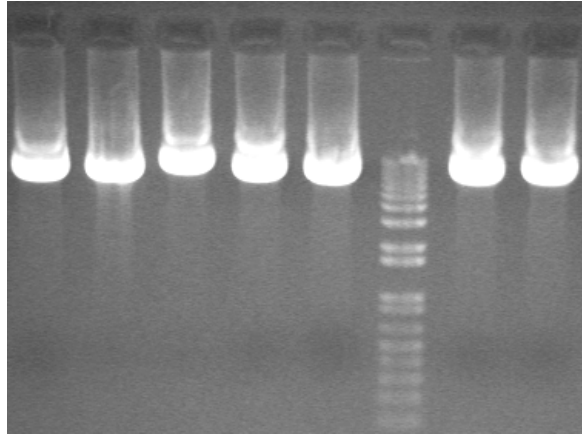


Figure 6.12– Check gel of purified plasmids containing aflR NotI prom and laeA NotI prom cloned into pTRAN3 1A vector in the direction of lacZ. Plasmid DNA is ready for transformation into *Aspergillus nidulans* strain.

Errors in this experiment

Initially, primers were created as such that the forward primer was 500bp upstream of the start site of the gene of interest and the reverse primer was just after the end of the stop site. Basically, the promoter region and the whole gene of interest was cloned into the vector, which if inserted – would not form a functional reporter gene because cloning the whole gene would result in a fusion protein between the gene of interest and lacZ or uidA.

Future Research

(1) Nutrient Regulation of Secondary Metabolite Production

Research Questions to explore

- (a) How does carbon/nitrogen source trigger/control/maintain gene expression and the production of secondary metabolites (specifically aflatoxin)?
- (b) How can the role of carbon metabolism (glucose vs. arabinose) and nitrogen metabolism (nitrate vs. ammonium) trigger/control/maintain gene expression and the accumulation of secondary metabolites (specifically aflatoxin)?
- (c) How does nutrient regulation control secondary metabolite production? Is the rationale due to: (i) Carbon/nitrogen metabolism? (ii) Signal transduction pathways? (iii) Energy status of the cell? (iv) Epigenetic regulation?
- (d) Why is there a differing effect when a rich growth medium is used vs. a minimal media? Is this effect a mimic of what is happening in ecology? The composition of minimal media is more relevant to what is actually happening in nature?
- (e) Does nutrient regulation play a direct role in activating *cre1bp*?
- (f) Is glucose a molecule for triggering signal transduction pathways or is glucose a molecule of metabolism? Is glucose directly affecting the signaling pathway, upon the addition of glucose – the effect should be relatively quick after its addition. But if it is a molecule for metabolism, the effect should take longer after its addition.
- (g) How is the effect of regulation different on solid vs. liquid media? Liquid cultures were created to optimize laboratory conditions but solid cultures will mimic environmental conditions more closely.
- (h) How similar is the regulatory mechanism for nutrients in *A. parasiticus* vs. *A. nidulans*?

Possible experiments to conduct

- (a) To characterize/quantitate the expression level of transcripts by real-time PCR (*aflR*, *nor-1*, *ver-1*, *omtA*). It is also possible to monitor/measure end products of primary metabolism (acetate, acetyl coA, pyruvate) over time points – 24hr, 48hr, 72hr.
- (b) Conduct the same set of experiment but adding glucose/arabinose/nitrate/ammonium at 36hr, then the same experiment but at 48hr.

- (c) Add end products of primary metabolism (acetate, acetyl coA, pyruvate) to cultures at 24hr and 48hr but do not add fresh nutrient source.
- (d) Add end products and fresh nutrient sources at 24hr, 48hr, 72hr.
- (e) Conduct the same sets of experiments on solid media.

These experiments might help characterize and correlate changes in substrate concentration, end product concentration and aflatoxin accumulation with changes in aflatoxin gene expression. The current research hypothesis states that when glucose/nutrient becomes limiting, aflatoxin gene expression increases and secondary metabolite production increases.

- (f) Observe the production of brown pigments in arabinose cultures in liquid media.
- (g) Repeat nutrient regulation experiments (glucose, arabinose, nitrate, ammonia) on *A. parasiticus* and compare the results from *A. nidulans*.
- (h) Repeat nutrient regulation experiments with solid/liquid media and rich/minimal media.

(2) Inhibitor Studies

Wortmannin

- (a) How does this inhibitor influence signal transduction pathways proposed in the model under initial studies (page - 15)?
- (b) We know that wortmannin affects intracellular cAMP, PKA activity and aflatoxin accumulation, but how does wortmannin affect PI3-kinase activity and phosphodiesterase activity?
- (c) Why is the influence of this inhibitor different under different carbon conditions, media composition and solid/liquid media?
- (d) Does wortmannin affect the role of CRE1-bp directly or indirectly possibly through its effect on protein kinase A (PKA) activity?
- (e) Does wortmannin alter the structure of chromatin through epigenetic regulation?

(3) Global Regulation

(The discussion under global regulation is part of a book chapter presentation written by Gary A. Payne and Jiujiang Yu)

There are currently two global regulators that we are interested in, *veA* and *laeA*. The function of *laeA* has been discussed above. *veA* functions in light-dependent conidiation

and has been shown to be a global regulator of secondary metabolism (Calvo, Bok et al. 2004) The deletion of *veA* causes a loss of secondary metabolite production, specifically sterigmatocystin. Subsequent studies have also shown that the deletion of *veA* causes a complete loss of aflatoxin production under both light and dark conditions. *veA* has also been shown to control the expression of *aflR* in *A. flavus*, *A. parasiticus* and *A. nidulans*. Thus, this global regulator has been shown to possess a strong correlation with secondary metabolite production.

The discussion of *veA* brings up more interesting questions about secondary metabolism. It is also found that secondary metabolism and conidiation appear to be genetically linked. Sclerotia are asexual survival structures for *A. parasiticus* that doesn't possess a sexual cycle, whereas cleistothecia are sexual survival structures for *A. nidulans* that possess a sexual cycle. Sclerotia/cleistothecia are produced to survive environmental extremes under the control of the circadian rhythm. The circadian rhythm is a biological clock that signals the production of these developmental structures under unfavorable environmental conditions. The circadian rhythm is a biological clock that provides an internal measure of external time. In *A. parasiticus*, experiments have been done and they show that the disruption of the aflatoxin polyketide synthase gene, *pksA* and the fatty acid synthase gene, *fas-1A* enhanced sclerotia production. The disruption of *veA* abolished sclerotia production and eliminated aflatoxin production.

The other global regulator, *laeA* has also been shown to regulate sclerotia development. The deletion mutant $\Delta laeA$ showed decrease in sclerotia production where as the overexpressed strain of *laeA* (OE:*laeA*) showed increased sclerotia production. Thus, there is evidence that sclerotia production and secondary metabolite production are closely tied and genetically linked, through a clock controlled signaling pathway.

Overall Research Application, Bigger Picture

Fungal Research and Implications in Bio-fuel Production

By understanding that different environmental conditions can trigger/manipulate and control the product of secondary metabolism, we can alter conditions to favor production of secondary metabolite. Using this understanding, we can manipulate different conditions that produce different end products of secondary metabolism. By optimizing conditions as well, we can optimize the production of secondary metabolites of choice.

Fungal Research and Food Safety

By understanding the molecular mechanisms by which aflatoxin biosynthesis is regulated and inhibited, we can design safe/novel approaches to control aflatoxin contamination in the field and during storage of crops. In the world today, 6.725 billion people depend on the research and development in science and the agricultural sector for the safety of their food supply (U.S. Census Bureau). By the year 2050, forty-two years from today, this number is estimated to rise from its present 6.725 billion to 9.1 billion (United Nations). Africa and South Asia, where large percentages of the poor live today and where future food production is of concern, face substantial challenges due to their current and projected increases in population. Thus, preventing food wastage from aflatoxin contaminated crops in developing countries especially will ensure a continuous supply of safe food.

Fungal Research and Food Waste Renewal

From the hypothesis that environmental stimuli (nutrient sources) can regulate the production of secondary metabolism, there is a possibility to use fungal systems to ferment food waste in order to generate renewable resources like energy. This means that the starting material given to the fungus can control/manipulate the variety of secondary metabolites which it produces. Utilizing fungal systems to complete this task is relatively cost efficient. Fungal systems are very versatile and they have a high adaptability rate. They can ferment almost any type of substance, and by understanding the basis of secondary metabolism, we can make use of this process to generate substances that will

be beneficial to us. One challenge that this technology might face is the separation of food waste that is generated. Food waste from school cafeteria, home kitchens and fast food restaurant can hopefully be used to generate renewable resources.

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Useful Links

Aspergillus flavus Genome Browser and information on life cycle of *A. flavus*
<http://www.aspergillusflavus.org/aflavus/>

Aspergillus nidulans Genome Browser
http://www.broadinstitute.org/annotation/genome/aspergillus_group/GenomeDescriptions.html#iAflavusi

USDA ARS website
http://fsrio.nal.usda.gov/document_reslist.php?product_id=69

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