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Identification of a critical gene in the dihydroergot alkaloid pathway

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**Thesis submitted
to the Davis College of Agriculture, Natural Resources, and Design
at West Virginia University**

in partial fulfillment of the requirements for the degree of

**Master of Science in
Applied and Environmental Microbiology**

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Division of Plant and Soil Sciences

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Abstract

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Ergot alkaloids, bioactive compounds produced by some species of fungi, have had significant impacts on agriculture and medicine. *Claviceps purpurea* and *Epichloë* spp. are ergot alkaloid producers associated with agricultural commodities and negatively affect humans and grazing animals, respectively. Another ergot alkaloid-producing fungus called *Aspergillus fumigatus* is ubiquitous in the environment; infections arising after inhaling the spores may kill

immunocompromised persons. Ergot alkaloids and their derivatives are utilized by the pharmaceutical industry for the development of drugs. Some of these drugs are derived from lysergic acid, whereas others are derived from dihydrolysergic acid. Lysergic acid derivatives have vasoconstrictive properties and are used as treatment of acute headaches and in obstetrics.

Dihydrolysergic acid derivatives are vasorelaxant and are the basis of drugs that help treat dementia, Alzheimer's, and Cushing's diseases. The ergot alkaloid pathway has largely been elucidated, but unknown steps remain, particularly in the dihydrolysergic acid branch. The gene encoding the enzyme that oxidizes festuclavine, a key intermediate in the dihydrolysergic acid branch, to fumigaclavine B is unknown. Based on DNA sequence analysis, we hypothesized that the *A. fumigatus* gene *easM* encodes this enzyme. To test this hypothesis we knocked out *easM* by inserting a hygromycin resistance gene in the middle of its coding sequence and transforming *A. fumigatus* with this construct. HPLC analysis showed that knock out of *easM* blocked the ergot pathway at festuclavine, and three downstream products (fumigaclavines B, A, and C) were eliminated. PCR results showed the hygromycin resistance fragment was present in the middle of *easM*-coding sequences in these transformants. Complementation, or reintroduction of the native non-disrupted *easM* gene restored the ability of the fungus to produce all downstream compounds. These results indicate that *easM* encodes the enzyme that oxidizes festuclavine to fumigaclavine B. The festuclavine-accumulating strain of *A. fumigatus* will be instrumental in future studies investigating the biosynthesis of dihydrolysergic acid derivatives, which are derived from festuclavine and are the basis for important vasorelaxant drugs.

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1 Overview

Ergot alkaloids are bioactive compounds that can be produced by several different species of fungi in the families Clavicipitaceae and Trichocomaceae. These fungi, collectively, can be found in a variety of environments. Some can live as saprotrophs, whereas others can infect a variety of hosts, including grasses and cereals, or humans and animals. The ergot alkaloid pathway for all ergot alkaloid-producing fungi appears to be shared in the early steps, but their divergence at the chanoclavine-I aldehyde branch point (Figure 1) leads to unique end products that are dependent upon the species of fungus producing them (Panaccione et al. 2012). Typical products of these pathways include clavines and lysergic acid derivatives. The synthesized chemicals are pertinent in medicine, as they are used in pharmaceuticals, and are of concern in agriculture due to their effect on grazing animals. Ergot alkaloid biosynthesis has been well studied but a few unknown steps remain thus, further understanding of these compounds can be of benefit for applications in medicine and agriculture in the future.

1.1 History and significance

Ergot alkaloid-producing fungi established their significance throughout history. Agricultural notoriety took root when grain crops, particularly rye, infected with *Claviceps purpurea* caused products made from the infected cereals to be unsafe to eat. A hard black structure called a sclerotium replaces seed within the flowering portion of the grass and was processed as grain until its deleterious effects were discovered. The mycotoxins produced within these hard overwintering structures are able to survive baking temperatures and digestive conditions, thus making them tenacious toxins (Merkel et al. 2012). Ergot poisoning or ergotism causes a variety of symptoms from hallucinations to gangrene of the extremities and convulsions

(Belser-Ehrlich et al. 2013). “St. Anthony’s fire” or ignis sacer derives its name from the burning sensations occurring in the extremities due to the vasoconstrictive activities of ergot alkaloids ingested from ergot-infected grains. Ergotism left such an impression on history that renaissance painters like Matthias Grünewald and Hieronymus Bosch immortalized the disease through art. Fortunately, the diversification of diets and processing or screening of grain has largely alleviated ergotism’s threat to consumers. Belser-Ehrlich et al. (2013) chronicle the major outbreaks of ergotism in humans since 1900 occurring in Russia in 1926-1927, England in 1927, Ireland in 1929, France in 1951, India in 1956-1957 and 1975, and Ethiopia in 1977-1978 and 2001. Luckily these outbreaks have not been equal in magnitude to those of the Middle Ages, and they were confined to remote areas or countries where regulations are not rigorous. Current regulations in developed countries around the world limit the amount of ergot that can be consumed by humans; for example, in the US the limit for ergot presence in rye cannot exceed 0.3 percent (Code of Federal Regulations 2001) and is lower for other products like oats, wheat, and barley.

Deleterious effects from ergot-producing fungi may also be experienced by livestock ingesting contaminated grasses or feed. *Epichloë* species, grass symbionts capable of producing ergot alkaloids, have been known to cause grazing livestock to experience unfavorable symptoms including vasoconstriction at the extremities resulting in gangrene (Schardl et al. 2006). *Epichloë* endophytes are known to produce four classes of alkaloids: ergot alkaloids (lysergic acid and ergovaline), indolediterpenes, pyrrolopyrazine, and aminopyrrolizidines that act as insecticidal compounds. Among these four classes, ergot alkaloids and indolediterpenes can affect vertebrates (Schardl and Phillips 1997). Coupled with environmental conditions, ergot toxicosis has varying effects on livestock. For example in heat stress conditions, animals that

ingest the toxins lose control of body temperature, water balance, experience necrosis of adipose tissue, and have the tendency to eat less food. Ingestion of toxins in cold conditions leads to gangrene. In general, toxin ingestion causes a reduction of lactation and fertility and, particularly in horses, miscarriages, fetal abnormalities, and stillbirths (Schardl and Phillips 1997). Economic losses due to livestock toxicosis may be ameliorated by using genetic techniques targeting toxin producing genes of mycobionts. Genetic engineering techniques are being explored, but since endophytes benefit their plant hosts, special attention needs to be paid to the acuteness of toxicosis in animals and environmental and genetic factors in selecting agronomically desirable endophytes that will maintain their endophyte niche but will not harm grazing livestock. Thus, there is still much to be explored regarding the control of toxin producing endophytes.

Ergot alkaloids' effects on history were not all negative; people have attempted to harness their biological activities for religious ceremonies and medicinal applications. In ancient Aztec cultures, priests would eat morning glory seeds with their associated alkaloid producing endophytes to induce psychoactive effects as a means of communication with the gods (Hofmann, Ratsch, & Schultes 1992). Albert Hoffman was the research chemist responsible for the discovery of lysergic acid diethylamide (LSD) and also investigated the psychoactive effects of seed endophytes and hallucinogenic mushrooms eaten in South America (Hoffman 1980). His studies indicated that the chemicals lysergic acid amide, ergonovine, and lysergic acid hydroxyethylamide (the main active component in the preparations ingested by the indigenous people of South America) are closely chemically related to LSD. Beyond the psychoactive effects, lysergic acid derivatives act on smooth muscle tissues which is why midwives in the Middle Ages utilized ergot alkaloids to aid in child birth; however, because concentrations of alkaloid were difficult to control in preparations and often caused complications they eventually

fell out of use (Tudzynski, Correia, & Keller 2001). With time, more became known about alkaloid activities and with it the ability to control dosage. Alkaloids began being incorporated into headache medications and a host of other drugs on the market today.

1.2 Ergot alkaloid-producing organisms

Aspergillus fumigatus is a common saprophyte in the phylum Ascomycota. *A. fumigatus* plays a role in an agricultural aspect: it spoils stored crops, such as animal feed especially in warm, moist conditions (Scudamore & Livesey 1997). *A. fumigatus* is ubiquitous due to its key role in environmental decay; thus, it can be found at especially high concentrations around composting facilities and other areas where there is a large amount of decomposing matter. *A. fumigatus* conidia are minute and very buoyant (Panaccione and Coyle 2005) allowing them to travel great distances, aiding the organism in spread and infection. The presence of *A. fumigatus* in air has been associated with respiratory ailments and allergic symptoms in humans. In immunocompromised persons it may manifest as invasive aspergillosis or systemic spread of infection, with a mortality rate of more than fifty percent. In healthy individuals, the immune system will typically clear the infection, but in some cases the fungus may colonize cavities within lung tissue or sinuses resulting in aspergilloma or fungus ball (Denning 1998).

A. fumigatus synthesizes alkaloids called clavines, or more particularly festuclavine, fumigaclavine A, fumigaclavine B, and fumigaclavine C, with fumigaclavine A or fumigaclavine C typically occurring as the end product in the pathway (Robinson and Panaccione 2012). Alkaloid production is associated with asexual reproduction, with alkaloid metabolites localized in the conidia (Coyle et al. 2007). *Aspergillus fumigatus* is an ideal candidate for the study of ergot alkaloid biosynthesis because it grows quickly in the saprophytic state in vitro yielding

measurable amounts of chemical. Its genome has also been sequenced, with numerous studies investigating genes within the ergot alkaloid gene cluster through knockout and complementation studies. Resulting knockout strains are important for serving as recipients for the expression of ergot alkaloid synthesizing genes of other fungi. This study follows suit with the knockout and complementation of a gene with an unassigned role in the ergot alkaloid gene cluster. Future studies will be able to utilize the knockout strain of *A. fumigatus* as a recipient for a gene of interest in an attempt to understand dihydroergot alkaloid biosynthesis.

Epichloë spp. (formally *Neotyphodium* spp.) are another genus of fungi in the family Clavicipitaceae that produce ergot alkaloids; they are grass symbionts that colonize above ground portions of plants, growing in the intercellular spaces. Tall fescue and perennial rye grass are two of the most commonly studied grasses relating to *Epichloë* spp. as symbionts. Symbiotic or endophytic relationships between grasses and *Epichloë* species are seed transmissible (Bacon & White 2000) meaning that the symbiotic fungus not only grows in the intercellular spaces of the plant but that it will grow into the developing seed for the purpose of dispersal and colonization when the new seed germinates. The mutualism provides the plant with drought tolerance, enhanced photosynthetic rate, and growth (Clay & Schardl 2002). Ergot alkaloid production in plants has also been shown to deter herbivory; a study found that rabbits preferred to eat grasses colonized by endophytic fungi that were alkaloid free versus grasses containing alkaloids or even endophyte-free grasses (Panaccione et al. 2006). Additionally, it has been well documented that biologically active alkaloids of the fungus have insecticidal activities, conferring protection to its host and increasing its competitiveness (Schardl et al. 2004). The lysergic acid derivatives produced by *Epichloë* spp. cause livestock feeding on these grasses to experience detrimental effects such as poor fertility, convulsions, poor weight gain, gangrene,

and even death (Schardl & Phillips 1997). Studying alkaloid producing endophytes is worthwhile to understand how they can be better managed out in the field to decrease their effects on the economic losses pertaining to grazing animals.

1.3 Ergot alkaloid cluster, pathway, and unknown steps

Ergot alkaloid synthesizing genes are found in clusters; however, the clusters vary depending on the organism (Figure 2). Genetic variation within clusters between species allows for the rich diversity in the ergot alkaloid pathway allowing some species to produce lysergic acid derivatives, dihydrolysergic acid derivatives, ergopeptines, or fumigaclavines (Figures 1 & 3). Arrangement of genes, variation in coding sequences for alleles, and even the presence or absence of some genes within clusters vary, and may be observed via sequencing of the ergot alkaloid clusters (Figure 2) (Schardl et al. 2013; Coyle and Panaccione 2005). For example, genes unique to the *A. fumigatus* cluster (*easN*, and *easL*,) are responsible for the production of different fumigaclavines (Figure 4). All of these genes have been sequenced, but some of their roles have not yet been assigned; this is the primary focus of this study.

Early steps in the ergot alkaloid synthesis pathway are evolutionarily conserved among alkaloid producing fungi (Figure 3). An early step in the ergot alkaloid pathway is the prenylation of tryptophan by DMATrp synthase, coded by the *dmaW* gene. Genetic and biochemical analyses demonstrated that it is the determining step in the ergot pathway for *A. fumigatus* (Coyle and Panaccione 2005). Knockout of *dmaW* in *A. fumigatus* completely eliminated all ergot alkaloids from the mutant, and the complementation restored production. The next important feature of the pathway is the chanoclavine-I aldehyde intermediate, at which the pathway diverges and varying terminal branch products are made according to individual

producers (Panaccione et al. 2012). Understanding which genes are responsible for each step in these pathways is critical for the study of the biosynthesis of the biologically important end products. Investigation of one of the remaining unknown steps, for which *easM* is hypothetically responsible, will help complete the pathway for the production of fumigaclavines in *A. fumigatus* and will be instrumental for future research of dihydroergot alkaloid biosynthesis.

1.4 Justification for choosing *easM* as a target gene

One of the few remaining unknown steps in fumigaclavine synthesis for *A. fumigatus* is the oxidative step responsible for modifying festuclavine into fumigaclavine B. Two candidate genes for this step are *easM* and *easK*, which are located in the ergot alkaloid gene cluster (Figures 2 & 4) and for which roles have not yet been assigned. Two pieces of evidence used to choose *easM* as the candidate gene for this study were as follows. First, the proposed structure for the protein coded by *easM* indicates that it is a P450 monooxygenase (Panaccione 2010). This putative function aligns well with the activity required to convert festuclavine to fumigaclavine B, which differs only by the presence of a hydroxyl group. Second, comparative genomics of *A. fumigatus* with *Penicillium commune*, organisms with similar clusters and similar ergot alkaloid profiles, indicates that the presence of *easM* and not *easK* correlates with the ability to produce ergot alkaloids (Panaccione 2010; Coyle & Panaccione 2005; Wallwey and Li 2011). Both *easM* and *easK* are present in *A. fumigatus* but *P. commune* only has *easM* while still retaining the ability to produce alkaloids (Wallwey and Li 2011). This leads us to hypothesize that *easK* is not essential for alkaloid production or that it may be redundant with *easM*. Both of these clues suggest that *easM* is the responsible gene for festuclavine oxidation to fumigaclavine B.

1.5 Benefits of research – agricultural and pharmaceutical implications

Pharmaceutically, derivatives of the ergot alkaloid pathway are important because they can be incorporated into drugs. Ergot alkaloids are similar in molecular structure to neurotransmitters that interact with serotonin, dopamine, adrenaline, and neurodreneline receptors (Belser-Ehrlich et al. 2013; Panaccione 2011). These similarities are attributed to the psychoactive and/or vasoconstrictive or relaxant properties associated with these mycotoxins. Mycotoxin agonism for serotonin-2A receptors is thought to be responsible for the hallucinogenic properties of ergot alkaloids (Vollenweider et al. 1998). Vasoconstrictive symptoms in the extremities are linked to alkaloids interacting with serotonin-1B/D receptors (Anderson et al. 1987). Vasoconstrictive properties of lysergic acid derivatives have been used to induce labor, terminate postpartum bleeding, and mimic dopamine (Hoffman 1980). Lysergic acid derivatives are currently ingredients in drugs that treat type II diabetes (Via et. al. 2010) and act as neurotransmitter agonists (Cabergoline) for treatment of pituitary disorders like Parkinson's disease. It is important to note that because alkaloids have affinities for many receptors and can act as agonist or antagonists, their biological activities are not simple and often associated with many side-effects (Panaccione 2010).

Dihydrolysergic acid derivatives differ from lysergic acid derivatives in that they lack a double bond in their D ring (Figure 5) and thus have vasorelaxant effects, opposite of those produced by lysergic acid derivatives. Dihydrolysergic acid derivatives are used in the treatment of disorders like dementia (Nicergoline) (Fioravanti & Flicker 2001), Alzheimer's, and Cushing's disease (Bracco et al. 2004). Patients with frequent, moderate headaches may receive a prescription from their provider for a nasal spray or injection called Migranal® a

dihydroergotamine (Valeant Pharmaceuticals, QC, Canada); tablets may also be available under the name Ergomar® and contain ergotamine (Rosedale Therapeutics, TN, USA) (Reddy 2013).

Thus, the production of dihydrolysergic acid by a novel fungus may have agricultural and pharmaceutical benefits. Dihydrolysergic acid-producing fungi in agriculture may counteract the vasoconstrictive effects of natural *Epichloë*-infected grasses. The development of a more efficient process for attaining dihydrolysergic acid, via biosynthesis rather than chemically, can be beneficial to the pharmaceutical industry by lowering costs.

2 Study Objectives

This study will elucidate an unknown step in fumigaclavine synthesis in *A. fumigatus*. I will test the hypothesis that the product of *easM* is necessary for oxidizing festuclavine to fumigaclavine B. One potentially significant outcome of this work would be the production of a strain of *A. fumigatus* that can be used for further studies on the biosynthesis of dihydrolysergic acid.

3 Materials and methods

3.1 Knockout construct preparation

The *easM* gene was PCR amplified from *A. fumigatus* template DNA in a reaction primed with *easM* forward and *easM* reverse primers (Table 1, Figure 6A). The EasM reaction consisted of 25 µL mixture containing: 1 X GoTaq Flexi buffer (Promega, Madison, WI), 1.5 mM MgCl₂, 200 µM each deoxynucleotide triphosphate, 1 µM of each designated primer, and 2.5 units GoTaq Flexi DNA polymerase (Promega, Madison, WI). The thermocycler program that carried out the procedure began with a denaturation step at 95°C for 3 minutes, then 35

cycles of: 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 3 minutes, and ending with a final extension of 72°C for 5 minutes. The *easM* fragment is 3,067 bases long, the length of which was confirmed by gel electrophoresis (Figure 6). The *easM* fragment was then ligated into plasmid pCR 2.1 to obtain a more concentrated product, and the ligation was transformed into *Escherichia coli*. *E. coli* colonies were grown on ampicillin-containing medium to select plasmid containing colonies. The presence of *easM* in the plasmid was determined by PCR primed with *easM* forward and *easM* reverse primers, in the same PCR program as described above. The product was then cleaned using a QIAquick column (Qiagen, Gaithersburg, MD).

To disrupt the *easM* coding sequence in the plasmid, it was digested with restriction enzymes *SmaI* and *ClaI* which cut out 1,091 bases located in the middle of the 3,067 base pair sequence of *easM*. The 1,443 base pair hygromycin resistance gene was amplified from template pCB1004 in a PCR reaction primed with *SmaHygF* and *ClaHygR* using the same thermocycler program as described above; recognition sites for *SmaI* and *ClaI* were included in the 5' ends of these primers and were used to introduce the hygromycin-resistance fragment into the gap of the *easM* plasmid by ligating the *SmaI-ClaI* cut hygromycin resistance fragment with the *SmaI-ClaI* cut *easM*-pCR 2.1 plasmid. The ligation was then transformed into *E. coli* and the resulting colonies were screened for the correct fragment via PCR using *easM* forward and reverse primers to visualize the 3,417 bp fragment of disrupted *easM* with the hygromycin fragment in the middle (Figure 6). As an additional check the fragment was digested again with restriction enzymes *SmaI*, *ClaI*, *EcoRI* to visualize that the cut portions corresponded to the predicted sizes. The insert of the final plasmid sample was amplified by PCR using *easM* forward and *easM* reverse primers and Phire Hot Start II polymerase (Thermo-Fisher Scientific, Waltham, MA) in a program starting with a 98°C denaturation for 30 seconds, followed by 35 cycles of: 98°C for 15

seconds, 65°C for 15 seconds, with a final extension at 72°C for 1 minute. The product was then cleaned using QIAquick column (Qiagen, Gaithersburg, MD).

3.2 Fungal transformation and complementation

The *easM*-HYG fragment was transformed into *A. fumigatus* by established methods (Coyle and Panaccione 2005) and grown on hygromycin selective medium. Only cells that took up the *easM*-hygromycin knockout construct were able to grow since they had the hygromycin resistance gene (Figure 6A).

To start the culture for transformation, *A. fumigatus* strain Af293 was grown on malt extract agar for approximately three days. Then, 10 mL of malt extract broth was used to create a spore suspension which was subsequently inoculated into an additional 20 mL of malt extract broth in a 50 mL flask and incubated overnight (approximately 16 hours) on an orbital shaker at 37°C and 80 rpm. Mycelium was pelleted in a 30-mL Nalgene Oak Ridge tube. Protoplasts were prepared by suspending the mycelial pellet in 15 mL of 0.7 M NaCl, 40 mg lysing enzyme (Sigma-Aldrich, Saint Louis, MO), and 1 g VinoTaste Pro (Novozymes, Switzerland) and allowing the mixture to incubate at 30°C for two hours. Protoplasts were separated from debris via filtration through a Miracloth (Calbiochem, San Diego, CA) funnel. Protoplasts were then washed by successively centrifuging (3,000 rpm for 5 min) and resuspending in the following solutions: 10 mL 0.7 M NaCl, 5 mL STC (1M sorbitol, 50 mM tris-Cl pH 7.4, 50 mM CaCl₂), and finally several hundred μ L of STC to achieve protoplast concentration of 5×10^6 protoplasts per 100 μ L. A solution of two parts 60% polyethylene glycol (PEG) and one part PEG amendments (1.8 M KCl, 150 mM CaCl₂, 150 mM Tris pH 7.4) was then added to comprise 1/5 of the volume. Aliquots of 125 μ L of protoplast-PEG solution were added to 1.5 mL sterile

tubes, along with 10 μ L of the *easM* knockout construct and then incubated on ice for 30 minutes. An additional 1 mL of PEG solution was added, the mixture was incubated at room temperature for 20 minutes and then plated onto Hygromycin (InvivoGen, San Diego, CA) selective medium.

A complementation assay was carried out to demonstrate that the phenotype of the *easM* knockout was due to the disruption at *easM* and not some other unrelated mutation in the colony. The wild-type allele of *easM* was first amplified from *easM* forward and *easM* reverse primers with Phire polymerase. The PCR product was cleaned up using Qiagen (Qiagen, Gaithersburg, MD) clean up kit. Next, a transformation was performed, as described above with Phleomycin (InvivoGen, San Diego, CA) resistance conferred by pBCphelo as the selectable marker onto Phleomycin (InvivoGen, San Diego, CA) selective medium.

3.3 PCR analysis of transformants

DNA preparations from each strain (wild type, *easM* knockout, and *easM* complemented) were checked via three PCR assays (Figure 6B). A wild-type screen primed with the *easM* forward and *easM* reverse primers checked for the size of the allele at the *easM* locus. A three prime screen with *easN*towardM with YG primers checked for presence of the 3' portion of *easM* adjacent to the hygromycin resistance gene. A five prime screen based on *easD*inF with HY primers checked for the presence of the 5' portion of *easM* adjacent to the hygromycin resistance gene.

3.4 Alkaloid analysis

To investigate the types of ergot alkaloids that accumulated in transformants, samples were analyzed by high performance liquid chromatography (HPLC) with fluorescence detection and liquid chromatography mass spectrometry (LC/MS).

Six replicate plates of *A. fumigatus* Af293 wild type, *easM* knockout, and *easM* complemented were grown on malt extract agar for two weeks. Cultures were extracted by removing a ~50 mm² sample area via the wide end of 1,000-μL pipette tip and suspending it in HPLC-grade methanol for an hour, rotating on a carousel. After centrifugation, supernatant (20 μL) of the extraction was analyzed via reverse phase HPLC. Samples were separated via C₁₈ column (Phenomenex Prodigy ODS3, 5-μm particle size, Torrance, CA). Ergot alkaloids were quantified by comparing peak areas to a standard curve of dihydroergotamine and normalizing to number of conidia extracted. Conidia in each extract were counted with the aid of a hemocytometer.

The uncharacterized chemical detected via HPLC eluting from the column at 55.9 minutes was explored with the aid of a Thermo Fisher LCQ DecaXP LC/MS. Six replicates of *A. fumigatus* Af293 wild type, *easM* knockout, and *easM* complemented were grown on malt agar plates for two weeks. Spores were suspended in 5 mL HPLC grade methanol and scraped from the agar plates with a spreader. Spores were allowed to spin on a carousel for three hours and then were centrifuged for ten minutes. Each sample yielded slightly over one mL of extract which was subsequently concentrated to 100 μL in a vacuum centrifuge. Of the resulting concentrate, 10 μL was then loaded to be analyzed by LC/MS. LC/MS analyses were conducted as described by Ryan et al. (2013).

3.5 Statistical analysis

Alkaloid concentrations were analyzed statistically with JMP software (SAS, Cary, NC). A Brown–Forsythe test was used to determine equality of group variances with $F > 0.05$ level. With equality of variances determined, an ANOVA was then used to compare quantities of each alkaloid among strains, with $p < 0.05$ indicating that chemical concentration varied by strain. This indication allowed for the subsequent Turkey-Kramer HSD test, to compare pairs to find significantly different means. Results reported in Table 2 display mean alkaloid concentrations $\pm$ standard error. Letters A, B, or AB within each column indicate the results of the Tukey-Kramer test, with same letters showing no difference between strains and different letters indicating a statistically significant difference.

4 Results

To test the hypothesis that *easM* is required for oxidation of festuclavine, *easM* was knocked out by replacing a large part of its coding sequences with a hygromycin resistance gene (Figure 6A). A complementation procedure was then carried out to restore the chemical profile and confirm *easM* function.

4.1 *easM* knockout

The native copy of *easM* in *A. fumigatus* was successfully knocked out through homologous recombination with the inactivated *easM*-HYG construct. Of the 54 transformant samples tested by HPLC, 12 showed an accumulation of festuclavine (the intermediate preceding the hypothesized EasM step) and loss of fumigaclavine C, the pathway end product (Figure 7). An unknown chemical, eluting from the column at 55.9 minutes, also appeared to be affected by

the *easM* knock out (Figure 7). Its identity was investigated via liquid chromatography mass spectrometry (LC/MS) in which a molecular ion of m/z 309.3 was detected (Figure 8). This molecular ion is consistent with the $[M+H]^+$ for prenylated festuclavine, an alkaloid previously detected in *A. fumigatus* and named 9-deacetoxyfumigaclavine C by Ge et al. (2009).

Three types of PCR assays showed that the *easM*-HYG knockout construct was, in fact, integrated at the *easM* locus. The first assay was primed with oligonucleotides flanking the intended site of integration (Fig. 6B). The fragment obtained from the *easM* knockout (KO) was larger than that obtained from the wild type (WT) *A. fumigatus* due to the replacement of a portion of the *easM* coding sequences with the hygromycin resistance genes (Figure 6A). The second (5' screen) and third (3' screen) assays were each conducted with one primer that flanked the 5' or 3' end of *easM*, respectively, and a second primer that annealed to the hygromycin-resistance gene (Figure 6A). The samples showed predicted size bands for each screen (Figure 6). This indicated that the wild type sample contained a functional copy of *easM* as expected, the knockout strain contained the *easM*-HYG construct which was integrated during fungal transformation, and that the complemented strain contained both the *easM* and *easM*-HYG fragments.

4.2 *easM* complementation

To demonstrate that phenotype of the *easM* knockout was due to the integration at *easM* and not any ectopic events, the *easM* knockout strain was complemented in trans with the wild-type allele of *easM*. Complementation resulted in restoration of the wild-type alkaloid profile in a qualitative sense (Figure 7). Integration of the complementing *easM* fragment was confirmed via PCR (Figure 6B). The presence of a fragment of wild-type length in the assay primed with

easM forward and *easM* reverse primers indicated the incorporation of the wild-type allele, whereas the presence of the longer fragment (typical of the *easM* knockout strain) indicated that the complementing allele had integrated ectopically. Thus the primers amplified the *easM* wild-type copy as well as the *easM*-HYG construct at the previously knocked out *easM* locus, resulting in fragments of 3 kb and 3.4 kb, respectively. Ectopic integration of the complementing allele also was supported by the observation of fragments typical of the knockout locus in the 5' flank and 3' flank PCR assays (Figure 6B).

4.3 Quantitative analysis of ergot alkaloids

Table 2 displays comparisons of mean alkaloid accumulation in each strain $\pm$ standard error. Festuclavine accumulated to significantly higher concentrations in the *A. fumigatus easM* knockout than in the *A. fumigatus* wild type and *easM* complemented strains ($P < 0.05$).

Fumigaclavine B production could be detected in *A. fumigatus* wild type and complemented strains but was eliminated by the *easM* knock out. Quantities detected in wild type and complemented strains do not differ significantly.

Fumigaclavine C was detected in the wild type and complemented strains but was lacking in the *easM* knockout strain. The increase in concentration in the complemented strain suggests a difference in regulation of the ectopically integrated gene.

The unknown chemical, hypothesized to be prenylated festuclavine (Figure 8) was detected in all three strains. Knock out of *easM* resulted in a significant increase in concentration of this compound. The complemented strain produced an intermediate amount of chemical, thus not differing significantly from either wild type or from the knockout strain.

5 Discussion

5.1 Role of EasM in the ergot alkaloid pathway

The pathway leading to the biosynthesis of lysergic acid is well established, but the pathway to dihydrolysergic acid-derived ergot alkaloids has not been elucidated. The objective of this study was to genetically manipulate steps in the ergot alkaloid pathway to prepare a strain of *A. fumigatus* suitable for future investigations of the biosynthesis of dihydrolysergic acid derived ergot alkaloids. Before exploring the dihydroergot alkaloid origin hypothesis, a festuclavine accumulator needed to be obtained. This was done by knocking out the relevant gene *easM* in the *A. fumigatus* ergot alkaloid pathway. Figure 4 illustrates the ergot alkaloid synthesis pathway in *A. fumigatus*. Genes responsible for the production of enzymes advancing each step of the pathway are indicated. However, the gene responsible for oxidizing festuclavine into fumigaclavine B had not been characterized. Dr. Panaccione and I hypothesized that the enzyme named EasM is responsible for oxidizing festuclavine to produce fumigaclavine B, which is then converted into downstream derivatives (Figure 4) based on its capacity to encode a P450 monooxygenase and its presence in the ergot alkaloid synthesis gene clusters of fumigaclavine producers. PCR and HPLC results along with the statistical analysis of this investigation described in the results section provide strong evidence that *easM* was successfully knocked out and complemented. These results indicate that the enzyme encoded by *easM* is necessary for oxidation of festuclavine into fumigaclavine B.

5.2 Differences in alkaloid quantity among wild type, knockout, and complemented strains

Table 2 shows a clear increase in the concentration of festuclavine and the putative prenylated festuclavine in the knockout mutant, this occurs because in the absence of EasM the

pathway cannot advance and the fungus accumulates festuclavine (Figure 4) a portion of which becomes prenylated by easL. Other data in Table 2 may raise questions regarding the difference in alkaloid quantities between the wild type and complemented strains of *A. fumigatus*. One reason for the difference with higher quantities of alkaloid (fumigaclavine C and unknown chemical) produced in the complemented strain, may be attributed to the fact that the integration of the wild type *easM* gene into the complemented strain could not be controlled with precision. During the transformation procedure relatively high concentrations of the wild type *easM* gene were introduced into the protoplast mixture to facilitate higher probability of complementation. Multiple copies of the wild type *easM* gene could have integrated into the genome. Additionally, the integration locus may have affected the quantity of chemical the complemented fungus was then able to produce, as some portions of the genome are more regulated than others. Thus the quantity of *easM* genes and their integration loci may be the explanatory factors for the difference in alkaloid quantity between the wild type and complemented *A. fumigatus* strains.

The transformation approach was chosen because it provided a reasonable strategy for analysis of gene function. The knockout construct was prepared in a relatively quick cloning and transformation procedure using a selectable marker, followed by a similar procedure for complementation. Phenotypes associated with ectopic integration of genes along with selectable markers could be reasonably differentiated via PCR and HPLC making this strategy a justifiable means for observing gene function.

The uncharacterized compound that accumulated to higher concentrations in the *easM* knockout strain of *A. fumigatus* (Figure 7 and 8) is hypothesized to be prenylated festuclavine. Rationale for this prediction is that festuclavine accumulates in the *easM* knockout strain due to the absence of a functional *easM* gene, the accumulated festuclavine may be utilized by an

enzyme called EasL. EasL is a prenyl transferase whose typical function is to prenylate fumigaclavine A converting it into fumigaclavine C (Unsöld and Li 2006; Robinson and Panaccione 2012). Because fumigaclavine A is missing, due to the *easM* knockout (Figures 4 and 6) EasL may accept other substrates, in this case festuclavine, prenylating it into the compound that eluted at 55.9 minutes (Figure 7) and had a molecular ion of 309.3 Da (Figure 8). The ability of EasL to prenylate a variety of ergot alkaloids has been observed in previous studies (Ge et al. 2009; Robinson and Panaccione 2014).

Fumigaclavines A and B are intermediates between festuclavine and fumigaclavine C (Figure 4). Fumigaclavine A was not detected (Figure 7 and Table 2) in any of the strains used in this study. Absence of fumigaclavine A may be explained via function of EasL which converts fumigaclavine A to fumigaclavine C (Unsöld and Li 2006; Robinson and Panaccione 2012). Small quantities of fumigaclavine B were detected because the enzyme EasN had not yet converted fumigaclavine B to fumigaclavine A.

5.3 Additional studies and future directions

A. fumigatus, a fumigaclavine producer, and lysergic acid producers like *Claviceps* spp. and *Epichloë* spp. differ in their allele for *easA* within the ergot alkaloid pathway (Coyle et al. 2010). *Epichloë* sp. Lp1 CloA typically accepts agroclavine as its substrate and turns it into lysergic acid through a series of oxidations and a double bond isomerization (Robinson and Panaccione 2014). Expressing *Epichloë* sp. Lp1 *cloA* in an *A. fumigatus* festuclavine accumulator may test whether *Epichloë* sp. Lp1 CloA is able to accept festuclavine and turn it into dihydrolysergic acid. Agroclavine and festuclavine differ in their structure only by the presence or absence of a double bond in the D ring (Figure 3). One hypothesis is that *easA* is the

only significant difference between dihydrolysergic and lysergic acid producers, with downstream enzymes in dihydrolysergic acid producers performing the same functions they do in lysergic acid producers.

Preliminary exploration of expressing an *Epichloë cloA* gene from an *easA* *A. fumigatus* promotor did not yield desirable results. The purpose of expressing *cloA* in an *A. fumigatus* festuclavine-accumulating strain was to test whether *Epichloë* sp. Lp1 native CloA, which typically modifies agroclavine into lysergic acid, would operate similarly in an *A. fumigatus* festuclavine-accumulating strain to produce dihydrolysergic acid. The core of lysergic and dihydrolysergic acid only differs by the presence of a double bond (Figure 5); festuclavine also possesses this same core (lacking a double bond) which may allow it to serve as a suitable substitute for the agroclavine substrate. Thus, CloA should add on functional groups in the two subsequent steps to make dihydrolysergic acid instead of the typical lysergic acid product (Figure 3). Additionally, evidence exists for permissiveness in substrate recognition in downstream enzymes; for example, LPS2 (Figure 3) in *Claviceps purpurea* has the ability to accept dihydrolysergic acid as well as lysergic acid as substrates (Riederer et al. 1996). This provides additional evidence that the enzymes of lysergic acid producing fungi have the ability to accept dihydrolysergic acid precursors. *Epichloë* sp. Lp1 *cloA* was expressed by fusing its coding sequences to the *A. fumigatus easA* promoter and the construct was transformed into the *A. fumigatus* festuclavine-accumulating strain. Conversion of festuclavine to lysergic acid was investigated via HPLC. Of the nineteen resulting transformants, integration of DNA was evident via PCR, but HPLC results did not indicate production of dihydrolysergic acid derivatives. Additionally, a feeding study testing the transformants ability to use exogenously supplied agroclavine and elymoclavine as substrates indicated that CloA was not utilizing the substrates

for conversion to downstream products. There are many reasons why the expression of *cloA* may not have worked: damaged DNA, integration of construct at a non-ideal site in the genome, lack of affinity for festuclavine (a dihydrolysergic acid precursor) as a suitable substrate, or perhaps a combination of native alleles (*easA* and *cloA*) are needed to make festuclavine work as a substrate for conversion to lysergic derivatives. Several fungi including *A. fumigatus* and *Epichloë* sp. have *easA* genes but their alleles differ. This may mean that the *easA* and *cloA* alleles from *Epichloë* may need to be transformed into *A. fumigatus*, as opposed to utilizing *cloA* from *Epichloë* sp. Lp1 and letting *A. fumigatus* utilize its own *easA*. Additional attempts have been made in utilizing *easA* and *cloA* from the same fungus, namely *Epichloë* but transformants did not result in desirable HPLC profiles. I suggest that future studies attempt to express other alleles of *easA* and *cloA* in the *A. fumigatus* festuclavine accumulator in an attempt to convert festuclavine to dihydrolysergic acid.

5.4 Conclusion

The ergot alkaloid synthesis cluster gene *easM* encodes a P450 monooxygenase that is necessary for oxidizing festuclavine into fumigaclavine B. Elucidating *easM* function is important as it adds to our knowledge of the ergot alkaloid pathway. Because *easM* had been sequenced but not yet characterized, this project provides evidence for *easM*'s function which had previously not been documented. Moreover, the engineering of a festuclavine accumulating *A. fumigatus* strain is instrumental in the next phase of research for the biosynthesis of dihydrolysergic acid. Dihydrolysergic acid derivatives are vasorelaxant and are the active compounds in drugs that help treat disorders like dementia, and Alzheimer's. Agricultural

applications for dihydroergot alkaloid producers which may counteract natural lysergic acid producing grass endophytes also exist.

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Figures and Tables

Figure 1: Divergence of the ergot alkaloid pathway at the chanoclavine-I aldehyde branch point. Note: Asexual *Epichloë* spp. were previously called *Neotyphodium* spp. The figure was modified from Panaccione et al. (2012).

A. *Aspergillus Fumigatus*

B. *Epichloë* sp. Lp1

Figure 2: Ergot alkaloid synthesis gene cluster of *A. fumigatus* (A) redrawn from Coyle and Panaccione (2005). *Epichloë* sp. Lp1 ergot alkaloid synthesis cluster (B) redrawn from Schardl et al. (2013). Compressed regions are designated with hash marks to allow for concise presentation. Arrows indicate direction of transcription. Functions have been assigned for all genes other than *easM* and *easK*.

Figure 3: Ergot alkaloid pathway reproduced from Robinson & Panaccione (2014).

Figure 4: Ergot alkaloid pathway in *A. fumigatus*, showing genes (above corresponding arrows) responsible for each step (Panaccione 2010).

Lysergic Acid

Dihydrolysergic acid

Figure 5: Lysergic acid and dihydrolysergic acid. The chemicals are the same except dihydrolysergic acid lacks a double bond in one of its rings.

Figure 6: (A) Schematic representation of *easM* locus in *A. fumigatus* before and after gene knock out. (B) PCR products of *A. fumigatus* isolate Af293 wild type, *easM* knockout, and *easM* complemented strains. Wild type screen is checking for the *easM* fragment. Three prime screen was primed with primers that anneal beyond the 3' end of *easM* and the middle of the hygromycin resistance gene. Five prime screen was primed with oligonucleotides that anneal before the 5' end of *easM* and in the middle of the hygromycin resistance gene.

Figure 7: High performance liquid chromatography (HPLC) results illustrating ergot alkaloid accumulation in wild type, *easM* knock out, and complemented *A. fumigatus* strains. Data were collected with a fluorescence detector (excitation 272 nm/emission 372 nm). Festuclavine eluted from the column at 41.9 minutes, fumigaclavine C eluted at 59 minutes, and a partially characterized compound (putative prenylated festuclavine; designated with an asterisk) eluted at 55.9 minutes.

Figure 8: Mass spectrum and predicted structure of chemical accumulating to higher concentrations in the *A. fumigatus easM* knockout strain.

Table 1 PCR primers used in this study and their products.

Primer combination	Primer sequence 5' → 3'	Product	Amplicon length (bp)
easMF + easMR	GCCATTCCTCCACTCTTCAG + CCAAAGAACATTCCTCAGCTC	<i>easM</i>	3,067
easNtowardM+ YG	GACTGCCAGTATCATCTACC + CGTTGCAAGACCTGCCTGAA	3' flank of integrated ko construct	2,057
easDinF + HY	ACTGCGCTACAGTCGATGTAAC + GGATGCCTCCGCTCGAAGTA	5' flank of integrated ko construct	2,916

Table 2 Mean alkaloid concentration (mean amol/spore $\pm$ standard error; n = 6) in *A. fumigatus* wild type, *easM* knockout, and *easM* complemented strains. Within each column, designation with different letters indicates significant difference ($\alpha=0.05$) in a Tukey-Kramer HSD test (or one-way ANOVA, when alkaloid detected in two strains).

Strain	Festucaclavine	Fumigaclavine B	Fumigaclavine C	Unknown ^a
<i>A. fumigatus</i> Af293 wild type	6.30 $\pm$ 4.92 A	0.58 $\pm$ 0.09 A	8.83 $\pm$ 1.37 A	1.19 $\pm$ 0.90 A
<i>A. fumigatus</i> Af293 <i>easM</i> knockout	33.59 $\pm$ 9.19 B	n.d. ^b	n.d.	8.39 $\pm$ 3.14 B
<i>A. fumigatus</i> Af293 <i>easM</i> complemented	13.91 $\pm$ 6.38 A	0.35 $\pm$ 0.05 A	23.12 $\pm$ 3.90 B	4.64 $\pm$ 3.72 AB

^a putative prenylated festucaclavine

^b not detected; limit of detection = 0.01 amol/spore.