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Ergot alkaloid synthetic capacity of *Penicillium camemberti*

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Thesis submitted to the Davis College of Agriculture, Natural Resources and Design at

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In partial fulfillment of the requirements for the degree of
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Abstract
Ergot alkaloid synthetic capacity of *Penicillium camemberti*

Samantha Fabian

Penicillium camemberti plays a major role in the ripening process of brie and camembert type cheeses. Investigation of the recently sequenced *P. camemberti* genome revealed the presence of a cluster of five genes previously shown to be required for ergot alkaloid synthesis in other fungi. Clustered with the five ergot alkaloid synthesis genes (aka *eas* genes) were two additional genes with that had the apparent capacity to encode enzymes involved in secondary metabolism. We analyzed samples of brie and camembert cheeses as well as cultures of *P. camemberti* grown under different conditions and were not able to detect any known ergot alkaloids, indicating the *P. camemberti* *eas* genes were either not expressed or encoded non-functional enzymes. We used a heterologous expression strategy to investigate the theoretical biosynthetic capacity of *P. camemberti*. Based on studies with the related ergot alkaloid-producing fungus *Neosartorya fumigata* (syn. *Aspergillus fumigatus*), the five known *eas* genes found clustered in the *P. camemberti* genome should give the fungus the capacity to produce the ergot alkaloid chanoclavine-I aldehyde. We used a chanoclavine-I aldehyde-accumulating mutant of *N. fumigata* as a recipient strain in which to express the two uncharacterized *P. camemberti* *eas* cluster genes (named *easH* and *easQ*) to create a functioning facsimile of the *P. camemberti* cluster. Expression of *easH* and *easQ* in the chanoclavine-I accumulating *N. fumigata* resulted in the accumulation of a pair of compounds of m/z 269.1 in positive mode LC-MS. Since this m/z is consistent with the mass of the isomeric pair of [rugulovasine A/B + H], we analyzed a culture of the related rugulovasine producer *Penicillium biforme* and found the same isomeric pair of analytes. Fragmentation of the analytes yielded fragments typical of those resulting from fragmentation of rugulovasine A/B. The deduced activities of the products of *easH* and *easQ* catalyze theoretical reactions that provide a reasonable pathway from the precursor chanoclavine-I aldehyde to the products rugulovasine A/B. When individually studied, the chanoclavine-I aldehyde-accumulating mutant of *N. fumigata* transformed with the *P. camemberti* *easQ* gene yielded a ratio of analytes at m/z 271.1 vs. m/z 255.1 that support the theorized mechanisms of *easH* and *easQ* to produce rugulovasines. The *P. biforme* genome contains a homologous *eas* cluster with only 12 mutations relative to that of *P. camemberti*. Eleven of the 12 mutations were investigated via complementation studies of the mutated genes (*dmaW*, *easC*, *easE*) using knockout strains of *N. fumigata*. The data indicate that *P. camemberti* has the genes to produce the ergot alkaloid rugulovasine A/B but that during domestication isolates that failed to produce alkaloids were selected for.

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Introduction/ Literature Review

***P. camemberti* history and importance**

Camembert type cheeses were thought to be first made in 1791 in the village of Camembert, near Normandy, France by Marie Harel (Spinnler and Gripon, 2004). While Camembert is a popular choice for U.S. consumers today, it holds historical significance in French culture. The WWI French troops were famously issued the cheese in their military rations and thus it gained popularity. The process of this making cheese is highly dependent on *P. camemberti*. Without the fungus, the cheese would not have the characteristic flavor and texture, as the mold plays a crucial part in the ripening process.

During the initial stages of the cheese making process, the milk is inoculated with *P. camemberti* as well as bacterial cultures and renin. As the cheese matures, the fungus colonizes the entire outside surface area giving the cheese its characteristic white felt rind. Perhaps the biggest contribution *P. camemberti* has to the ripening process is its ability to oxidize lactate, as the lactate is the main food source for the fungus (McSweeney, 2004). As a result of the lactate metabolism, gradients are established that help with the transport of other compounds from the middle of the cheese to the surface rind. *P. camemberti* oxidizes lactate to CO₂ and O₂ and over time results in increasing the pH of the rind of the cheese (McSweeney, 2004). This deacidification leads to the creation of a pH gradient from the cheese center to the rind which allows lactate to move to the rind surface, where it is oxidized by *P. camemberti*. The increase in pH on the rind also creates a calcium phosphate gradient, which allows migration of calcium and phosphate from the center to the cheese surface. The proteolysis by *P. camemberti* on the rind creates an excess of ammonia, which diffuses

into the cheese giving it its distinct odor. The initial oxidation of the lactate of the cheese is key in its production as it continues until all of the available lactate is metabolized.

The degradation of protein in camembert cheeses is very complex and involves many different microorganisms including lactic acid bacteria. However, *P. camemberti* contributes to the proteolysis of the cheese by making aspartyl and metalloproteinases, acid carboxypeptidase and an alkaline aminopeptidase (McSweeney, 2004). The aspartyl proteinases acts on α -casein, while the acid carboxypeptidases work on β -casein, hydrolyzing the Lys97-Va98I, Lys99-Glu100, and Lys29-Ile30 bonds (McSweeney & Fox, 2004). In the process of lipolysis, the milk itself contains lipolytic agents such as lipoprotein lipase. The renin coagulant and the starter microorganisms can also provide lipases that can metabolize the lipids present. *P. camemberti* has been found to oxidize linoleic and linolenic acids with lipoxygenases and hydroperoxide-lipases. It is capable of producing 9-, 10-, and 13-hydroperoxy acids from polyunsaturated fatty acid oxidation (Spinnler and Gripon, 2004). However, there is minimal lipolysis occurring in the cheese as the high lipid concentrations give the cheese its creamy texture.

Gene clusters and secondary metabolites

Fungi have interesting clusters of genes that encode the ability to produce secondary metabolites, or chemical compounds that can be beneficial in some way for the fungi. These clusters can code for catabolic pathways, activated to allow the fungus to survive under low nutrient/competitive environments by using low molecular weight nutrients as energy source (Keller and Hohn, 1997). They also code for enzymes that act as catalysts of biosynthetic pathways producing secondary metabolites (Keller and Hohn, 1997). While there are many, some common examples include penicillin and ergot alkaloids. The secondary metabolites can be beneficial or sometimes harmful to

plants, humans and animals. Secondary metabolite production is controlled by the activation/deactivation of the transcription of certain genes that are found in a cluster (Brakhage and Schroeckh, 2011). These gene clusters are unique to the chemical compound being produced but can be found in an array of different fungi. On another note, the discovery of these gene cluster does not mean the fungus is producing the metabolite, but rather that it has the potential to produce it.

Examples of secondary metabolites produced by *Penicillium* species

Penicillium species are producers of many secondary metabolites such as the well-known antibiotic penicillin. *P. camemberti* has been known to produce acetaldehyde, benzaldehyde, 3-methylbutanal and 1-octen-3-ol to inhibit the bacteria *Listeria monocytogenes* and *Salmonella typhimurium* (Larsen and Knochel, 1997). It is believed that the production of these chemicals is beneficial to *P. camemberti* as it reduces the competition of these potentially pathogenic gram negative/gram positive bacteria by inhibiting their growth. Additionally, *P. roqueforti* the fungus associated with the production of blue/roquefort cheese has been known to make antibiotics such as penicillin, as well as mycophenolic acid and patulin (Neilsen et al., 2006). *P. roqueforti* is also known for making ergot alkaloids festuclavine, isofumigaclavine A and isofumigaclavine B (Neilsen et al., 2006). Festuclavine is also made by *P. crustosum*, *P. palitans* and *P. oxalicum* (Kozlovsky, 1999) Therefore, it is not uncommon for *Penicillium* species to produce ergot alkaloids.

Ergot alkaloids

Ergot alkaloids are most famously produced by the fungus *Claviceps purpurea* and also are produced by a large range of other fungi including *Epichloë* spp. and *Neosartorya fumigata* (Panaccione and Coyle, 2005). They can also be produced by

certain *Penicillium* spp. that were previously discussed (Kozlovsky, 1999). Ergot alkaloids can be viewed as beneficial or harmful depending on the dose and context. Ergot alkaloid derivatives can be used directly or often are modified to serve as pharmaceuticals as well as illegal drugs, such as lysergic acid diethylamide or LSD. The most popular legal ergot alkaloid-derived drugs produced are for treating migraines, depression, prolactin disorders, gynecological issues, dementia and Parkinson's (Hulvova et al., 2013). The known fungal producers of these alkaloids are grown under optimal alkaloid production settings and then the alkaloids are extracted for use. However, ergot alkaloids can be harmful if consumed without any chemical modification or in uncontrolled doses. Ergot alkaloid poisoning, or ergotism, can cause a wide range of symptoms from hallucinations, gangrene, burning skin, neurological diseases and even death. Ergotism can occur in both humans and animals. Today, the incidence of ergotism is uncommon due to modern farming practices, but high levels of ergot alkaloids can still be found in the grasses of less advanced farming systems. Historically, ergotism was a widespread problem that was referred to as *St. Anthony's Fire* that swept across Europe and the early American colonies. The largest epidemic of ergotism occurred in 994 AD in Aquitaine, France where around 40,000 people died as a result (VanDongen and DeGroot, 1995).

Mechanism of action

The above-mentioned benefits and dangers of ergot alkaloids can be explained by the mechanism in which they affect the body. Because they have a similar chemical structure to the neurotransmitters dopamine, serotonin and norepinephrine, ergot alkaloids interact with the receptors for those neurotransmitters (Tudzynski et al., 2001). Figure 1 highlights the similarity among an alkaloid and three neurotransmitters.

However, the way the alkaloids interact with the neurotransmitters depend on the alkaloids structure and the particular sub-type of receptor. In some cases, ergot alkaloids act as agonists, whereas in other cases they are antagonists. Adding different chemical substituents to C-8 of the ergoline ring gives the alkaloid different affinities toward the different neuroreceptors (Schardl et al., 2006). Simple peptide ergot alkaloids have a high affinity for adrenergic receptors and thus are responsible for the vasoconstrictive effects. However, the clavines have a high affinity for serotonin receptors and therefore responsible for the anti-serotonergic action. Some ergot alkaloids can also have an effect on prolactin release and are therefore manipulated for pharmaceutical use for conditions like galactorrhea (Schardl et al., 2006).

Chemistry of ergot alkaloids

Chemically, the backbone of the alkaloids consists of 3,4-substituted indole derivatives with a tetracyclic ergoline ring structure (Tudzynski et al., 2001). There are three group classes of ergot alkaloids: clavines, lysergic acid amides, and ergopeptines based on their level of complexity and additional groups (Panaccione, 2005). The alkaloids are synthesized from the amino acid tryptophan and then metabolized into various pathway end products via enzymatic reactions. The enzymes are coded for by ergot alkaloid synthesis or *Eas* genes.

Genes of the ergot alkaloid synthesis cluster

The publicly available genome of *P. camemberti* features five ergot alkaloid synthesis (*eas*) genes in its genome: *dmaW*, *easC*, *easE*, *easF*, and *easD*. In *N. fumigata*, these genes code for the enzymes that catalyze synthesis of DMAPP and tryptophan into chanoclavine-I aldehyde (Figure 2; Figure 3). More specifically, the *dmaW* gene encodes for the enzyme dimethylallyltryptophan synthase which converts

tryptophan and DMAPP to dimethylallyltryptophan or DMAT (Wang et al., 2004; Coyle and Panaccione, 2005). The *easF* gene is responsible for coding for the enzyme dimethylallyltryptophan-N-methylase which converts DMAT to N-Me-DMAT (Rigbers and Li, 2009). Genes *easC* and *easE* code for the enzymes catalase and chanoclavine-synthase, respectively (Lorenz et al., 2010; Goetz et al., 2011). While the exact mechanism is unknown, it is known that they work together to form chanoclavine-I (Goetz et al., 2011). The enzyme chanoclavine-I dehydrogenase coded by the *easD* gene then converts chanoclavine-I to chanoclavine-I aldehyde (Wallwey et al., 2011). This gene cluster and production of chanoclavine-I aldehyde from tryptophan and DMAT is common to most ergot alkaloid producers such as *Claviceps/Epichloë* and *Neosartorya/Penicillium*. At this point, the synthesis of the specific ergot alkaloids produced by the different species is catalyzed by different enzymes in lineage-specific branches.

Susceptible to evolution adaptability and mutability of *Penicillium* species

Multiple lines of evidence have led to the idea that *Penicillium* species are readily susceptible to evolutionary processes. One such example of evolutionary adaptation is the concept of horizontal gene transfer between different species of *Penicillium*. *P. camemberti* and *P. roqueforti* are known to undergo these gene transfers frequently with each other. While these two species of *Penicillium* are both primarily grown on dairy substrates and are used to produce cheese, phylogenetically they are not closely related (Cheeseman et al., 2014). However, they were found to undergo the same horizontal gene transfers of two genes that give the fungi fitness and competitive advantages in a cheese environment (Cheeseman et al., 2014).

***Penicillium biforme*: A close ancestor**

While *P. camemberti* is a characteristically white mold found exclusively in cheese products, it is a descendent of *Penicillium biforme* and *Penicillium fuscoglaucum* which are blue/gray type molds (Ropars et al., 2015). Because of its close relation to *P. camemberti*, *P. biforme* was also studied as part of this thesis project. *P. biforme* naturally occurs in cheese substrates but has been found to be a contaminant in other dairy-based foods and juices as well. *P. biforme* is a known producer of the ergot alkaloid rugulovasine (Dorner et al., 1980). Like other known ergot alkaloid producers, my preliminary investigation of its publicly available genome indicated it has the first five ergot alkaloid synthesis genes along with two additional genes, *easH* and *easQ* (Figure 3). Thus, the ability of *P. camemberti* to produce ergot alkaloids was studied due to its close relation to *P. biforme* and its importance in the production of camembert cheese.

Rationale and hypothesis:

Due to the previously studied activities of the five ergot alkaloid synthesis genes found in *P. camemberti*, the ability of this fungus to produce ergot alkaloids was researched. Considering that other *Penicillium* species are capable of producing alkaloids and rapidly evolving, it is reasonable to hypothesize that *P. camemberti* has the capacity to produce ergot alkaloids. With *P. biforme* phylogenetically being a recent ancestor to *P. camemberti*, it could be that during domestication mutations caused this gene cluster to no longer function in *P. camemberti*.

Methods

Characterization of *P. camemberti* and *P. biforme* ergot alkaloid gene clusters

The ergot alkaloid gene cluster in *P. camemberti* was found by comparing individual *eas* genes from *N. fumigata* to the NCBI database. Contigs containing *P.*

camemberti and *P. biforme* eas gene clusters were downloaded and additional genes were annotated by BlastX searching individual, contiguous 5-kb fragments.

HPLC analysis

Fungi were cultured on malt extract agar (6g malt extract, 1.8 maltose, 6g dextrose, 1.2g yeast extract per liter of distilled water) for 7 to 10 days, and samples were prepared for HPLC analysis by cutting a 1 cm by 1 cm piece of fungus-colonized medium and adding it to 400 μ L of HPLC grade methanol. Samples were rotated end-over-end (15 rpm) for 35 minutes and centrifuged at 13,000 rpm for 10 minutes. One hundred μ L of supernatant was placed into an HPLC sample vial and 20 μ L was analyzed for ergot alkaloids on reverse phase liquid chromatography using a C18 column and the protocol outlined in Panaccione et al. (2012). Analytes were monitored by fluorescence with excitation at 272 nm and the emission 372 nm. The area under the peaks were measured and converted into ng/mL using a standard curve generated from prior HPLC data.

Liquid chromatography mass spectrometry (LC-MS) analysis

Samples were prepared for LC-MS by using 5 mL of 100% methanol to remove all spores and hyphae from the malt extract agar cultures grown for 7-21 days. Two mL of the harvested spore suspension was centrifuged at 13,000 rpm for 10 minutes. The supernatant was collected and concentrated to 100 μ L in a speedvac. Ten μ L of the extract was then analyzed with a Thermo Fisher LCQ LC-MS according to methods described by Ryan et al. (2013) except the gradient program initiated with 86% mobile phase A (5 parts acetonitrile + 95 parts 0.1% formic acid) and 14% mobile phase B (75% acetonitrile arts acetonitrile + 95 parts 0.1% formic acid) and increased linearly to 100% mobile phase B over 20 minutes. Flow was maintained at 100% B for 5 min,

before initial conditions were restored. Flow rate was 200 $\mu\text{L}/\text{min}$. The peaks in the chromatograms of interest were measured using the LC-Qual browser and analyzed for the observation of conversion ratios.

Fungal transformation protocol

Cultures for preparing protoplasts for transformation were grown as follows. A 2-cm x 2-cm sample of fungus–colonized malt extract agar of various strains of *N. fumigata* was used as inoculum to start cultures in 50 mL of malt extract broth. Cultures were incubated overnight at 37°C, 80 rpm. To prepare protoplasts, the broth was replaced with 15 mL 0.7 M NaCl that included 40 mg lysing enzyme (Sigma-Aldrich, St. Louis, MO) and 1 g VinoTaste Pro (Gusmer Enterprises Inc., Mountainside, NJ). The enzyme-mycelium mixture was then incubated for 2 hours at 37°C and 80 rpm. The mixture was filtered through a miracloth (EMD Chemicals, Gibbstown, NJ) lined funnel in order to separate the protoplasts from the hyphae and other debris. The protoplasts were pelleted by centrifuging for three minutes at 3000 rpm. The supernatant was decanted, and then the protoplasts were resuspended with 10 mL 0.7 M NaCl and then re-pelleted. The process was then repeated but with resuspension in 5 mL STC (1 M Sorbitol, 0mM Tris pH 7.4, 10m 6M CaCl_2). The protoplasts were then resuspended in a final volume of STC that allowed for a protoplast concentration of 5×10^6 protoplasts in 100 μL STC. Two volumes of 37.5% PEG 8000 and one volume of PEG amendments (1.8 M KCl, 150 mM CaCl_2 , 150 mM Tris pH = 7.4) were mixed together in order to prepare a polyethylene glycol (PEG) solution. A quarter of the final protoplast volume was calculated, and then the prepared PEG was added in that amount. Ten μL of plasmid DNA or PCR product was added to the 125 μL of the final protoplast mixture, which was incubated on ice for 30 minutes. One mL of the PEG solution was added to

the cells and then the mixture was incubated at room temperature for 20 minutes. The solution was then divided among three Petri dishes and mixed with 15 mL molten TM102 media, which corresponds to M102 medium as defined by Rykard et al. (1982) but containing 1 M sucrose. After the agar had solidified, 15 mL of molten TM102 mixed with 800 µg/mL phleomycin (InvivoGen, San Diego, CA) was added on top of solidified layer. The plates were incubated at 37 °C for three days.

Preparation of gene constructs for fungal transformation

A typical PCR reaction was comprised of 11 µL distilled/deionized water, 5 µL 5x Phusion HF buffer (100 mM KCl, 20 mM Tris-HCl pH 7.4, 1.5 mM MgCl₂; Thermo Scientific, Waltham, MA), 4 µL 1.25 mM dNTPs, 1.25 µL 0.5 mM forward primer, 1.25 µL 0.5 mM reverse primer, 2 µL of template DNA and 0.5 µL of Phusion high-fidelity polymerase (Thermo Scientific). The reactions underwent the same general cycle, with an initial denaturation at 98°C for 30s, followed by 35 cycles of 98°C (15 s), annealing temperature (as defined by primer pair in Table 1) (15 s), and 72°C for and extension time as defined in Table 1, followed by a final extension at 72°C for 60 s. The primers, annealing temperatures, and extension times are included in Table 1. The PCR products were cleaned using the Zymogen DNA Clean & Concentrator kit (Zymo Research Corp., Irvine, CA). The cleaned products observed via gel electrophoresis in 0.8% agarose.

Plasmid preparation

Various restriction enzyme sites were included in the primers used to PCR amplify different gene fragments. The PCR products with restriction enzyme sites were cleaned using the Zymogen DNA Clean & Concentrator kit (Zymo Research Corp., Irvine, CA) and then digested with the appropriate restriction endonuclease for 90 minutes at 37°C. The following solution mix was used for all digestions: 6 µL of cleaned

PCR products with restriction sites, 10 µL distilled water, 2 µL of CutSmart buffer (New England BioLabs, Ipswich, MA) and 1 µL each of the corresponding restriction enzymes associated with the specific gene products. A previously prepared pBCPhleo PCR product was also digested under the same conditions with the same set of restriction enzymes used for the individual gene PCR product. The digested products were cleaned using the Zymogen DNA Clean & Concentrator kit. The cleaned digests were loaded on a 0.8% agarose gel and the intensities of the products were recorded. A ligation reaction was created based on the observed intensities of the digested fragments, with 8 µL of the mixture being comprised of a ratio of the amplified gene digest and the digested pBCphleo; along with 1 µL of 10x ligation buffer and 1 µL of ligase (New England BioLabs, Ipswich, MA). The solution was incubated at 16°C overnight. The ligation was then transformed into a Novagen NovaBlue™ Competent *E. coli* cells (EMD Biosciences Inc, Darmstadt, Germany) and grown on LB + chloramphenicol (50 µg/mL) agar. Resulting colonies were isolated and grown in 2 mL of LB + chloramphenicol broth and incubated at 37°C overnight. The cells were pelleted and the plasmids were purified using the Zyppy™ Plasmid Miniprep kit (Zymo Research Corp., Irvine, CA).

Expression of of *easQ/easH* Fusion PCR product in *N. fumigata*

The *easH* and *easQ* genes of *P. camemberti* were individually PCR amplified using primer sets 13 and 14 (Table 1). The cleaned PCR products were then then attached to a *N. fumigata easA/easG* bidirectional promoter via fusion PCR. The previously generated *easH*, *easQ* and a previously generated *N. fumigata* promoter PCR products were used as template DNA and the primer set 15 (Table 1). The 4145-bp fusion PCR product along with a previously created pBCphleo linear plasmid were

co-transformed in an equal molar concentration into a previously created chanoclavine-I aldehyde accumulating *easA* ko strain of *N. fumigata* (Coyle et al., 2010).

Transformants were analyzed by HPLC with fluorescence detection and LC-MS.

Observation of the individual function of *P. camemberti easH* and *easQ*

P. camemberti easH and *easQ* gene fragments with restriction enzyme sites were PCR amplified using primers 11 and 12, respectively (Table 1). The *easH* fragment contained restriction enzyme sites for *NotI* and *BamHI*, while the *easQ* PCR product contained sites for *NotI* and *SpeI*. The PCR products were digested with their corresponding restriction enzyme set, cleaned, and then ligated with pBCphleo. The resulting plasmid was used for fungal transformation into the *N. fumigata easA* knockout strain (Coyle et al., 2010). The successful transformants were analyzed by HPLC with fluorescence detection along with LC-MS.

Functional analysis of *P. camemberti easC*, *easE* and *dmaW*

The functionality of *P. camemberti easC*, *easE* and *dmaW* was tested by using those genes in attempted complementation of previously characterized *N. fumigata* knockout mutants. The *P. camemberti* gene fragments were PCR amplified using primer sets 1, 2 and 3 (Table 1) that included restriction enzyme sites. The *easC* fragment contained sites for *BamHI* and *EcoRI*, *easE* featured sites for *NotI* and *SpeI*, and *dmaW* had restriction enzyme sites for *NotI* and *BamHI*. The cleaned PCR products were digested with the indicated restriction enzyme set, cleaned and then ligated with similarly-digested pBCphleo. The resulting plasmids were used for fungal transformation into previously made *easC*, *easE* and *dmaW* knockout strains of *N. fumigata* (Goetz et al., 2011; Coyle & Panaccione, 2005). The transformants were plated on malt extract agar plates and analyzed by HPLC with fluorescent detection.

Extraction of messenger RNA and preparation of cDNA

Cultures of *P. camemberti* NRRL 874 were grown as surface cultures on malt extract broth for three days at room temperature. A 100 mg sample of the fungal mat was then frozen with liquid nitrogen and ground into a powder with a mortar and pestle. RNA was then extracted by following the instructions of the Qiagen RNeasy Plant kit (Qiagen Company, Hilden, Germany). cDNA was obtained by reverse transcribing the mRNA with SuperScript IV reverse transcriptase (Thermo Scientific, Waltham, MA). The cDNA was then used as template for primer sets 4, 5, 6, 7, 8, 9 and 10 (Table 1). PCR products were analyzed via gel electrophoresis.

Analysis of different *Penicillium* spp. isolates and cheese prepared with *P. camemberti* or *P. biforme* for ergot alkaloids

Cultures of *P. camemberti* NRRL 874, NRRL 875, NRRL 66321 and *P. biforme* (*P. camemberti* NRRL strain 885) were grown on malt extract agar at 22°C for seven days. Cultures were also analyzed via LC-MS analysis.

Samples of brie and camembert cheese bought commercially and those made in lab were also analyzed using the same HPLC and LC-MS detection methods outlined above. The lab generated cheese samples were made with mesophilic cheese culture (New England Cheesemaking Supply Co., South Deerfield, MA) and *P. camemberti* NRRL 874 and *P. biforme* NRRL 885 individually as the ripening fungi. Curds were allowed to mature for eight weeks.

Results

***Penicillium camemberti* has an ergot alkaloid synthesis (*eas*) gene cluster with a unique combination of *eas* genes**

Prior research has established that the genes *dmaW*, *easF*, *easC*, *easE*, and *easD* (Figure 3) encode the enzymes that catalyze the reactions that transform DMAPP

and tryptophan to chanoclavine-I aldehyde (Figure 2). Genomes of other fungi were investigated for contigs that contain the *eas* genes through sequence queries. The search revealed that *P. camemberti* contains an *eas* cluster containing homologs of *dmaW*, *easF*, *easC*, *easE*, and *easD* (Figure 3; Table 2). This same observation has recently been noted by Martin et al. (2017). Further analysis of the *P. camemberti* contig that contained the *eas* gene cluster revealed a copy of the gene *easH*, alleles of which have two very different functions in two different fungi (Havemann et al., 2014; Jakubczyk et al., 2015) and also a copy of a novel gene with undetermined functionality, referred to as *easQ* (Figure 3; Table 2). The presence of this combination of genes indicated that *P. camemberti* has the genetic potential to produce chanoclavine-I aldehyde, with the possibility that the products of *easH* and *easQ* genes modify chanoclavine-I aldehyde into a more complex ergot alkaloid.

***Penicillium camemberti* alleles of *easH* and *easQ* contribute to ergot alkaloid synthesis**

Roles in ergot alkaloid biosynthesis for *P. camemberti* genes *easH* and *easQ* were derived via transformation studies. The *easH* and *easQ* genes of *P. camemberti* strain NRRL 874 were individually PCR amplified and then attached to a *N. fumigata* *easA/easG* bidirectional promoter via fusion PCR. The *easH/easQ* fusion PCR product was co-transformed along with pBC-Phleo into a chanoclavine aldehyde accumulating *easA* ko strain of *N. fumigata*. Eight fungal transformant colonies grew on 400-μg/mL phleomycin plates and were then analyzed by HPLC with fluorescence detection to observe alkaloid accumulation. While chanoclavine aldehyde was detected, no novel or additional alkaloids were observed in the HPLC analysis. DNA was extracted from the transformants and studied via PCR to investigate the presence of the construct (Figure 4). A transformant that contained *easH* and *easQ* was further analyzed by LC-MS to

compare its metabolite profile with that of the recipient *easA* ko strain of *N. fumigata* (Figures 5 and 6). The transformed fungal strain was found to produce chanoclavine-I (Figure 5C) and a pair of analytes with m/z values of 269.1288 (Figure 6). A pair of compounds with the same molecular ion and retention times was produced by *Penicillium biforme* (Figure 6), a close relative and proposed ancestor to *P. camemberti* but not by the chanoclavine aldehyde-accumulating *easA* ko strain of *N. fumigata* used as a recipient in this experiment (Figure 5). Prior research has established that *P. biforme* produces of the isomeric pair of ergot alkaloids called rugulovasine A and rugulovasine B, the $[M+H]^+$ for each of which has a calculated m/z of 269.1285. Further comparison of *P. biforme* and the *easH/easQ*-transformed *easA* ko strain of *N. fumigata* via fragmentation analysis revealed that the two produced identical fragments, the m/z values of which matched with the fragments produced upon fragmentation of rugulovasine (De Medeiros et al., 2015) (Figure 6). The data indicate that that the products of *easH* and *easQ* act on chanoclavine aldehyde to produce rugulovasines A and B.

Individual functions of *easH* and *easQ*

While together *easH* and *easQ* were observed to produce rugulovasines from chanoclavine-I aldehyde, the individual functions of the products of these genes were also explored. *P. camemberti easH* gene under the control of the *N. fumigata easA* promoter was transformed into the chanoclavine-I aldehyde-accumulating *easA* ko strain of *N. fumigata*. Ten transformants containing the *easH* expression construct grew on media containing 400 $\mu\text{g/mL}$ phleomycin, indicating that those cultures had taken up the phleomycin resistance-conferring construct. PCR analysis of genomic DNA template demonstrated that the transformant contained the introduced *easH* gene (Figure 7A).

RT-PCR analysis of cDNA template demonstrated that the *easH* mRNA was transcribed in the transformant (Figure 8A), indicating that the gene was being expressed.

Sequence analysis and prior research indicates that both EasH and EasQ may be oxidases. Thus it is likely that they may be adding an oxygen to chanoclavine-I aldehyde. This addition would result in the m/z value observed for chanoclavine-I aldehyde increasing from 255 to 271. Therefore, special attention was paid in LC/MS analyses for appearance or increase in analytes with m/z of 271. The transformed strain was analyzed for accumulation of ergot alkaloids by HPLC with fluorescence detection and by LC-MS, but no novel peaks or ratios were observed as compared to the profile of the *N. fumigata easA* knockout. *EasQ* was studied in the same manner, by transforming the chanoclavine-I aldehyde accumulating *easA* ko strain of *N. fumigata* with the *P. camemberti easQ* gene linked with the *N. fumigata easA* promoter. PCR and RT-PCR analyses indicated the gene was present and transcribed (Figure 7B; Figure 8B). HPLC with fluorescence detection did not reveal the presence of any novel metabolites when only a single gene was expressed. However, when analyzed on LC-MS it was observed there was a large peak at m/z in the *easQ* transformant. This m/z 271 peak was also observed in the *N. fumigata easA* knockout and the *easH* transformant, but in very small quantities. Thus, it was hypothesized that the m/z 271 product may be the oxidation product of the m/z 255 analyte (chanoclavine-I aldehyde) and that the ratios of these analytes may differ between strains. The areas under the EIC peaks were then measured and analyzed using ANOVA. It was found that the ratios were significantly different based on the measured strain ($p=0.0017$). A Tukey's multiple comparison test showed that the *N. fumigata easA* with the *easQ* gene had a much higher m/z 271 to m/z 255 ratio than the *N. fumigata easA* knockout strain ($p=0.002$) or

the *easH* transformed *N.fumigata easA* knockout ($p=0.007$), supporting the hypothesis that EasQ oxidized chanoclavine-I aldehyde to a molecule with m/z 271 (Figure 9; Table 3).

Investigation of the lack of accumulation of ergot alkaloids in *P. camemberti* strain NRRL 874

Possible reasons for lack of accumulation of rugulovasine in *P. camemberti* include lack of expression of *eas* genes, misprocessing of *eas* gene transcripts, or null mutations in individual *eas* genes. Whether the *eas* genes of *P. camemberti* were transcriptionally active was investigated in qualitative RT-PCR analyses. Messenger RNA from *dmaW*, *easF*, *easC*, *easE*, *easD*, *easH* and *easQ* were present in reverse transcriptase-PCR analyses indicating that those genes are being transcribed in *P. camemberti* strain NRRL 874 when grown in malt extract broth (Figure 10). Additionally, sequencing results proved that the mRNAs were being processed properly, as the cDNA contained open reading frames corresponding to those for functional *eas* genes from *N. fumigata*.

The possibility that individual *eas* genes of *P. camemberti* were mutated was investigated by comparing the *P. camemberti eas* gene cluster to the functional *eas* cluster of *P. biforme*. The contig of *P. biforme* that contains its *eas* gene cluster was obtained from NCBI GENbank (accession number CBXO010000115.1) and compared to the *P. camemberti eas* gene cluster (GenBank accession CBVV010000119.1) by Blast analysis. The two *eas* clusters were nearly identical, with the exception of five differences in the coding regions of *eas* genes and seven intergenic differences or polymorphisms (Figure 11). Each of the five polymorphisms that occurred within coding sequences resulted in changes in amino acids in four different enzymes (Figure 11C).

Two amino acid changes occurred in EasE, one in EasC, one in DmaW, and one in EasQ.

In order to test functionality of the *P. camemberti* *eas* genes, three of the four genes that contained SNPs relative to the established functional homologs in *P. biforme* were transformed into previously constructed *N. fumigata* knockout strains; the function of EasQ was evident from its co-expression with EasH in *N. fumigata* strain *easA* ko. Transformation with *P. camemberti* *dmaW* and *easE* complemented the respective *N. fumigata* mutants. Transformed strains that contained the *dmaW* or *easE* constructs each accumulated fumigaclavine C, indicating that the *P. camemberti* allele restored the *N. fumigata* ergot alkaloid pathway (Figure 12; Figure 7C-D). Attempts to complement an *easC* knockout mutant of *N. fumigata* (Goetz et al., 2011) did not result in a strain that expressed *easC*, thus the function of the EasC enzyme could not be assessed.

Analysis of potential ergot alkaloid accumulation in cheese

Four different wheels of Camembert cheese were cultured and ripened with *P. biforme* NRRL strain 885 and *P. camemberti* NRRL strain 874 for eight weeks (Figure 13). Samples were analyzed by LC-MS along with a sample of *P. biforme* grown on malt extract agar for comparison. Rugulovasine was not detected in any camembert cheese sample. Samples of commercial cheese ripened with *P. camemberti* also were investigated and found to be free of rugulovasine and chanoclavine-I.

Discussion

My data illustrates that *P. camemberti* previously had the genetic capacity to produce ergot alkaloids such as chanoclavine-I and rugulovasines A and B. My research also has identified a new ergot alkaloid biosynthesis gene (*easQ*), provided evidence for a novel functionality of an allele of the gene *easH*, and indicated a biosynthetic pathway for the rugulovasine class of ergot alkaloids. The transformation of *easH* and *easQ* into the chanoclavine-I aldehyde-accumulating *easA* knockout strain of *N. fumigata* induced the fungus to produce the alkaloids rugulovasines A and B. The biochemical activity and sequence in which *easH* and *easQ* functioned were also studied. Typically, the *eas* genes present in *N. fumigata* control the reactions to turn tryptophan and dimethylallylpyrophosphate to chanoclavine-I aldehyde and then to festuclavine and fumigaclavine A, B, and C (Figure 2) (Robinson and Panaccione, 2015). While it is clear from the expression studies in *N. fumigata* that the *easH* and *easQ* genes of *P. camemberti* are functional, *P. camemberti* does not produce ergot alkaloids in culture nor were the alkaloids detected in any cheese samples obtained or created. To explain this lack of ergot alkaloids in *P. camemberti* in light of the genetic data, we looked at evolutionary data, taking into account the closely related rugulovasine-producing strains of *Penicillium* spp. Differences in the *eas* gene clusters between *P. camemberti* and *P. biforme* were studied through complementation and mRNA expression studies to investigate a possible explanation. It was found that *P. camemberti* encodes functional *easE* and *dmaW* genes but further investigation of the *easC* gene is needed to fully understand if all the genes in the ergot alkaloid synthesis gene cluster are functional.

Rugulovasines

Structurally, rugulovasines are classified as indole alkaloids in the ergot family that contain a spirocyclic butyrolactone subunit (Zhang et al., 2013). Rugulovasines are unique as they occur as two racemic isomers, A and B, that are capable of being interconverted by heating in polar solvents (Meurant, 2012). Ergot alkaloids are great chemical structures to undergo biohalogenation as they contain nitrogen in their chemical structure (Gribble, 2008). Thus, it there are also naturally-occurring chlorinated and brominated forms of rugulovasine (De Medeiros et al., 2015).

While the health effects of the rugulovasines are largely understudied, a few studies indicate they may induce negative health consequences. Previous research showed that ingestion of rugulovasines by day-old poultry caused acute toxicity effects (Dorner et al., 1980). In fact, this toxicity was used as a bioassay to purify the rugulovasines. It has also been reported that the rugulovasines caused decreased blood pressure when administered to cats (Meurant, 2012). Our LC-MS data indicate that *P. bifforme* and the *easH/easQ*-transformed *easA* ko strain of *N. fumigata* are capable of producing both rugulovasine A and B (Figure 6). This is evident as the fragmentation spectra for both rugulovasine A and B match the fragmentation spectra of rugulovasines from previous studies (De Medeiros et al., 2015). However, the chlorinated and brominated forms of rugulovasine were not detected when analyzed on LC-MS based on the absence of analytes of the predicted masses established in analyses by De Medeiros et al. (2015). It is also pertinent to note that while rugulovasines were detected in large quantities in our LC-MS data, they were not detected on the HPLC with fluorescence detection (monitoring at excitation/emission wavelengths of 272 nm/372 nm or 310 nm/410 nm, respectively). Future steps may be

to monitor different combinations of excitation and emission wavelengths in order for the rugulovasines to fluoresce for detection on the HPLC. It is also possible that the particular structure of these compounds prevents or quenches fluorescence.

Proposed mechanism of rugulovasine biosynthesis

We hypothesize that EasQ, predicted by sequence analysis to be an aldehyde dehydrogenase, oxidizes chanoclavine-I aldehyde to the corresponding carboxylic acid (Figure 14). As the name indicates, an aldehyde dehydrogenase is capable of acting on aldehydes, oxidizing the aldehyde to a carboxylic acid. The aldehyde dehydrogenase family of enzymes are NADP-dependent that share the basic structural and functional attributes that allow them to catalyze the oxidation of a number of different aliphatic and aromatic aldehydes (Lindahl, 1992). Evidence that the *P. camemberti* *easQ* gene is similar yet different from the other forms of aldehyde dehydrogenases can be observed from the phylogenetic and sequence match data (Figure 14B). In fact, a close homolog of *easQ* with two introns in identical position and phase is found in the *P. camemberti* genome and has close homologs in several other members of the Trichocomaceae, including *N. fumigata*. The data favor that *easQ* acts as the nomenclature indicates, turning chanoclavine-I aldehyde into chanoclavine-I acid (Figure 15). Analysis of the transformed chanoclavine-I aldehyde accumulating *easA* knockout strain of *N. fumigata* with the *easQ* gene on LC-MS supports this proposed mechanism as copious amounts of an analyte with the mass of [chanoclavine-I acid + H]⁺ (*m/z* 271) was detected (Figure 9). Whereas small quantities of this same metabolite were observed in the *N. fumigata* *easA* knockout strain, the ratio of chanoclavine-I (resumed substrate) to the *m/z* 271 analyte (presumed product) increased significantly upon expression of *easQ*. These results supported the hypothesis that EasQ may be acting on chanoclavine-I aldehyde

and converting it to the corresponding carboxylic acid. The amounts of the m/z 271 analyte compared to chanoclavine-I were also compared to the levels of these analytes found in the *easA* knockout strain of *N. fumigata* with the *easH* gene as well as the chanoclavine-I aldehyde accumulating *easA* knockout strain of *N. fumigata* (Figure 9). While there were trace amounts of the presumed chanoclavine-I acid found in the chanoclavine-I aldehyde accumulating *easA* knockout strain of *N. fumigata*, the increase in the analyte of this mass in the *easQ*-transformed strain as well as the ratios between the aldehyde and the proposed acid are indicative of the actions of EasQ (Figure 15). Furthermore, Blastp search has indicated that there is a distant *easQ* homologue present in *N. fumigata* as well as in *P. camemberti*. The other *P. camemberti* homologue, which is 58% identical in amino acid sequence to the product of the *easQ* gene used in this study, may be the parent gene from which *easQ* was neofunctionalized (Figure 14 A,B). Thus, the product of this gene in *N. fumigata* may be responsible for the small background quantities of the analyte with the mass of chanoclavine-I acid in *N. fumigata*. However, the data strongly favors that the *P. camemberti* EasQ is capable of converting chanoclavine-I aldehyde to chanoclavine-I acid (Figure 9).

EasH, a predicted dioxygenase, would oxidize the aromatic carbon customarily labeled as carbon 10 in chanoclavine-I aldehyde or perhaps that same carbon in the corresponding carboxylic acid, chanoclavine-I acid. Dehydration of the 10-hydroxylated chanoclavine-I acid would result in the formation of rugulovasine A (Figure 15). The literature supports that this proposed mechanism is possible but novel, as the product of no other form of the *easH* gene has acted on this proposed substrate. Jakubczyk et al. (2015) proposed three mechanisms by which EasH of the fungus *Aspergillus japonicus*

might oxidize the very same carbon in a slightly different ergot alkaloid precursor to result in formation of cycloclavine. That study outlined three potential mechanisms that may be encoded for by the *easH* gene (Figure 16). The first two outlined mechanisms propose that EasH may be abstracting a hydride ion (Figure 16A), or abstracting a hydrogen ion from its substrate (Figure 16B). Figure 16C shows that EasH may be hydroxylating or halogenating its substrate. Hydroxylation is the mechanism that is consistent with our hypothesis for *P. camemberti* EasH, as it would explain hydroxylating the chanoclavine-I acid produced from the actions of *easQ*

A study with another allele of *easH* from *Claviceps purpurea* stated that *easH* has the ability to code for enzymes that can hydroxylate amino acids that are in the first position of ergopeptines. The study also found that the product of that particular allele of *easH* was capable of cyclolizing the ergopeptine classes that are naturally occurring (Havemann et al., 2014). Figure 13C and D illustrates the phylogenetic relationships between the *easH* alleles from these fungi as well as those other fungi that share a similar *easH* gene with *P. camemberti*. The phylogenetic clustering of *P. camemberti* *easH* with those involved in cycloclavine biosynthesis support the hypothesis that they have similar activities on a similar substrate.

If EasH were to act on chanoclavine-I aldehyde (as opposed to its acid derivative described above), we propose that the intermediate would essentially be chanoclavine-I aldehyde with an additional oxygen at carbon 10, thus making its m/z around 271. While the *easH* gene alone was successfully transformed into the chanoclavine-I aldehyde-accumulating *easA* knockout strain of *N. fumigata* (Figure 6B), when analyzed on LC-MS there were no notable traces of this proposed intermediate. It is possible that EasQ needs to act on chanoclavine-I aldehyde first before EasH can undertake its

oxidation mechanisms (Figure 15). It may also be that intermediate is unstable in this form and therefore does not stay in solution long enough to be detected. The fact that the production of rugulovasine was detected when both *easH* and *easQ* were expressed supports the notion that they work together in catalyzing chanoclavine-I aldehyde into rugulovasine.

Ancestral species of *P.camemberti* are rugulovasine producers

Phylogenetic analysis shows that *P. biforme*, *P. fuscoglaucum* and *P. commune* are perhaps the ancestral strains of *P. camemberti* (Ropars et al., 2015; Polonelli et al., 1987). *P. biforme*, *P. fuscoglaucum* and *P. commune* are known as common contaminants of cheese and other food products. The close phylogenetic relationship between *P. biforme*, *P. fuscoglaucum* and *P. camemberti* was confirmed by using 17 different microsatellite markers (Giraud et al., 2009). *P. biforme* and *P. commune* are known for producing rugulovasines (Kozlovsky, 1999).

There are two chemotypes of *P. commune*, with Chemotype I being associated as a producer of cyclopenoxoic acid and rugulovasine and chemotype II producing fumigoclavines. *P. camemberti* is thought to have descended from *P. commune* chemotype I or the one that is known for producing rugulovasines (Frisvad and Filtenborg, 1989). This hypothesis is supported by data from Polonelli et al. (1987) who analyzed what appeared to be a *P. camemberti* mutant that produced the same alkaloids as *P. commune*. It was determined that this “mutant” was actually in a *P. commune*, thus *P. commune* must be the ancestor of *P. camemberti* (Polonelli et al., 1987). All of these phylogenic lineages favor the notion that the ancestor to *P.*

camemberti had the genetic capacity to produce rugulovasine but at some point in the domestication of the commercial strain the fungus lost that ability.

Lack of ergot alkaloid production in *P. camemberti*

While the data suggest that the products of the *P. camemberti* *easH* and *easQ* genes act on chanoclavine-I aldehyde to produce rugulovasines, ergot alkaloids were not detected in any strain of *P. camemberti*. Interestingly enough, *P. camemberti* does transcribe all of the *eas* genes in the ergot alkaloid synthesis gene cluster under the cultural conditions commonly used in our analyses (Figure 9), which involved culturing the fungus in malt extract medium without agitation at ambient temperature. Sequence data collected on these transcripts indicated proper processing of mRNAs, eliminating that step as a reason for the lack of ergot alkaloids in *P. camemberti*. *P. biforme* shares a similar ergot alkaloid gene cluster with *P. camemberti* with the exception of a few nucleotide differences (Figure 11). Because there were minor differences between the *P. camemberti* *easC*, *easE* and *dmaW* genes in comparison to the *P. biforme* *eas* genes, these genes were selected for complementation studies. The *P. camemberti* *dmaW* and *easE* genes were found to be fully functional, as they were able to be transformed into the *N. fumigata* the respective knockout strain and restore the fungus's ability to produce festuclavine C (Figure 12). However, attempts to express the *P. camemberti* *easC* failed to result in accumulation of *easC* mRNA. One potential reason for this observation is that expression of *P. camemberti* *easC* in *N. fumigata* may require placing the coding sequences of the gene under the control of an *N. fumigata* promoter.

Implications

The collected data has shown an ancestral capacity of *P. camemberti* to produce ergot alkaloids. The data suggested potential mechanisms of the novel *P. camemberti*

easQ gene and the novel allele of *easH* and the role they play in the production of rugulovasines. Moreover, while it may be reassuring to know that domesticated strains of *P. camemberti* used for the production of camembert-type cheeses did not produce any detectable ergot alkaloids, some *Penicillium* strain associated with dairy products may have the ability to produce rugulovasines (Polonelli et al., 1987). While there is little known about how rugulovasines may affect human health, the literature does indicate that it is a compound that may have detrimental health impacts if prolonged exposure persists (Dorner et al., 1980; Meurant, 2012).

It is possible that *P. biforme* may produce rugulovasines as a means of defense of resources and that *P. camemberti* does not need this defense as it is only found on cheese products which have a defined microflora and are cultivated hygienically. Another theory is that historically the rugulovasines may have made consumers of the cheese sick, therefore strains of *P. camemberti* that were unable to produce these alkaloids were selected. It is possible that there may be more to this observation and therefore it should be further studied. Future studies to try to explain this phenomenon would include sequencing and comparing the *eas* gene cluster of *P. fuscoglaucum* and *P. commune* to try to pinpoint where this divergence may have occurred. Also, it is pertinent to note that while *P. biforme* produces rugulovasines in culture, no alkaloids were detected when *P. biforme* was grown on cheese substrates.

Figures

Figure 1: Chemical structures of neurotransmitters dopamine, serotonin and norepinephrine on the left. These neurotransmitters interact with neuroreceptors to elicit a neuronal response in the body. Chemical structures of ergot alkaloids chanoclavine-I aldehyde and rugulovasine A are pictured on the right. Ergot alkaloids have a similar chemical backbone as the neurotransmitters, thus they can interact with the neuroreceptors as well.

Figure 2: Series of reactions catalyzed by the *eas* genes converting tryptophan and N-methyl DMAT to chanoclavine-I aldehyde. This pathway is common to most ergot alkaloid producers, with chanoclavine-I aldehyde acting as the branching point into more specialized alkaloids

Figure 3: **A:** The ergot alkaloid synthesis gene cluster from *P. camemberti*. Genes *easC*, *easF*, *easE*, *dmaW*, and *easD* are also found in *N. fumigata*. The combination of *easH* and *easQ* are novel to *P. camemberti* and *P. biforme*. **B:** Ergot alkaloid gene cluster found in *P. biforme*. **C:** gene cluster found in *N. fumigata*. While the *eas* gene cluster is more extensive in *N. fumigata*, it shares five of the same genes among *P. camemberti* and *P. biforme*.

Table 1: Primer combinations, annealing temperature, and extension time for the various PCR reactions.

Primer Combination	Primer sequences from 5' to 3'	Product	Size (bp)	Annealing Temp. (°C)	Ext. Time (s)
1	CTCAGAATTCGAGCTGGTCCGCTTCAGG + CTACGGATTCGTCGCTTGATGGCTGTGATGTAACG	<i>easC</i> with <i>Bam</i> HI and <i>Eco</i> RI restriction enzyme sites	3070	67	90
2	CAACGCGGCCGCAAGAGGATAGAGCTTTCAGCTGG + CAGAACTAGTGGTGATTCAACGAGGCCCATGG	<i>easE</i> with <i>Not</i> I and <i>Spe</i> I restriction enzyme sites	3642	67	90
3	CATCGGATCCGGCCAACATGACTCCACGGC + CTACGCGGCCGCGTGTAACTATGGAGGATGACAAGC	<i>dmaW</i> with <i>Not</i> I and <i>Bam</i> HI restriction enzyme sites	2686	67	90
4	CGCATCGGGTATTGGTGCCG + CTAGCCACATCCTCGGGCTC	<i>easD</i> cDNA gene product	670	62	45
5	GGACTTCCCAACCATGATCAG+CGAAGGAGGTAGGATGGCCAC	<i>dmaW</i> cDNA gene product	1282	62	45
6	GGAGTCATGTTGGCCATCTCGC + GTCCCACTGCCGCTTCAGC	<i>easE</i> cDNA gene product	1570	62	45
7	GGAATCCGGCACTGGATTGAAC + CTCTCAATCCCGCCGACCC	<i>easF</i> cDNA gene product	853	62	45
8	GCCTGGAGCAAGTCAAGTTCTCA + CTGATTGACGATTCTCTCCACC	<i>easC</i> cDNA gene product	1323	62	45
9	GAGTAGGCACTCCGCACCATGACGATTTCGAATTGCGCCAAC + CCTTGATGGGATACACAGTTGG	<i>easH</i> cDNA gene product	951	64	45
10	GTACTTGGTGGATTAGAAGCAATGTGTGAGACATCAATTGATCTGAC + GACTAGGTCAAGCATGACTGGC	<i>easQ</i> cDNA gene product	1659	64	45
11	GTATACTAGTGAATCCGCAATGAATCTGCAGG+CAGAGCGGCCGCTC TATGTGAAGCATGCGGGAATG	<i>easQ</i> with <i>Spe</i> I and <i>Not</i> I restriction enzyme sites	2967	62	90
12	ACCGGATCCGTGCCGTAGTCCTATACTAAG + CCACCGCGGTGGCGGCCGCTATTTGCTCACTTTGGCTTGCATG	<i>easH</i> with <i>Bam</i> HI and <i>Not</i> I restriction enzyme sites	2213	62	90
13	GTCAGATCAATTGATGTCTCACACATTGCTTCTAATCCACCAAGTAC + CCACCGCGGTGGCGGCCGCTATTTGCTCACTTTGGCTTGCATG	<i>N. fumigata</i> promotor + <i>easH</i>	2228	64	90
14	GTTGGCGCAATTGAAATCGTCATGGTGCGGAGTGCCTACTC + GGGGATCCACTAGTTCTAGAGCGGCCGCTCTATGTGAAGCATGCGG GAATGG	<i>N. fumigata</i> promotor + <i>easQ</i>	2754	64	90
15	CCACCGCGGTGGCGGCCGCTATTTGCTCACTTTGGCTTGCATG+ GGGGATCCACTAGTTCTAGAGCGGCCGCTCTATGTGAAGCATGCGG GAATGG	<i>easQ</i> + <i>N. fumigata</i> promotor + <i>easH</i>	4145	64	90

Table 2: Ergot alkaloid synthesis genes found in *P. camemberti* and their similarity to genes found in other known alkaloid producing fungi. The *P. camemberti* genome was obtained through NCBI and the individual genes were compared through NCBI Blastx. The function of these genes and percent identity are shown below.

Gene	% Identity	Species	Function	Accession number
<i>easQ</i>	97%	<i>Aspergillus flavus</i>	aldehyde dehydrogenase	XP_002376976.1
<i>easH</i>	99%	<i>Byssosclamyces spectabilis</i>	Phytanoyl-CoA dioxygenase	GAD93023.1
<i>easC</i>	86%	<i>Penicillium roqueforti</i>	Catalase haem-binding site	CDM36675.1
<i>easF</i>	93%	<i>Penicillium roqueforti</i>	SAM-dependent methyltransferase, EasF-type	CDM36676.1
<i>easE</i>	94%	<i>Penicillium roqueforti</i>	Berberine/berberine-like	CDM36677.1
<i>dmaW</i>	93%	<i>Penicillium roqueforti</i>	Tryptophan dimethylallyltransferase	CDM36678.1
<i>easD</i>	99%	<i>Aspergillus lentulus</i>	chanoclavine-I dehydrogenase	GAQ03955.1

Figure 4: *P. camemberti easH* and *easQ* were both present in the transformed *N. fumigata easA* ko fungus.

Figure 5. Extracted ion chromatogram m/z 269.2 from mass spectrometry analysis of *N. fumigata easA ko* (A), the *N. fumigata easA* knockout transformed with *easH* and *easQ* (B), and *P. bifforme* (C).

Figure 6: Mass spectrometry fragmentation patterns of rugulovasine (De Medeiros et. al., 2015) and the *N. fumigata* *easA* ko transformed with *easH* and *easQ*.

Figure 7: PCR evidence illustrating that fungal transformations were successful. Primer sets for specific amplification of the genes were used in conjunction with the transformed *N. fumigata* strains. **A)** presence of *easH* in the *N. fumigata easA* ko strain. **B)** presence of *easQ* in the *N. fumigata easA* ko strain. **C)** presence of *dmaW* in the *N. fumigata dmaW* ko strain. **D)** presence of *easE* in the *N. fumigata easE* ko strain.

Figure 8: cDNA of the *easH/easQ* transformed *N. fumigata easA* knockout. **A)** shows presence of *easH* while **B)** shows *easQ*. Primers for *easQ* flanked an intron, and the size (and DNA sequence) of the PCR product demonstrate it was derived from processed mRNA..

Figure 9: 271.1/255.1 m/z ratio differences observed in the LC-MS data of *N. fumigata easA ko*, *N. fumigata easA ko+easH* and *N. fumigata easA ko+easQ*. Brown-Forsythe analysis indicates significant differences between A and B as indicated by p-value further outlined in Table 3.

Table 3: Calculated p-values by Brown-Forsythe analysis that show that there is a difference in ratio of 271.1/255.1 m/z based on the strains analyzed. Asterisks indicate significance.

Strains analyzed by LC-MS	p-Value
<i>N. fumigata easA ko+ easQ</i> vs. <i>N. fumigata easA ko</i>	0.002*
<i>N. fumigata easA ko+ easQ</i> vs. <i>N. fumigata easA ko+ easH</i>	0.007*
<i>N. fumigata easA ko+ N. fumigata easA ko +easH</i>	0.673

Figure 10: RT-PCR of *dmaW*, *easF*, *easC*, *easE*, *easD*, *easH* and *easQ* transcripts from *P. camemberti* NRRL strain 874 cDNA. All seven genes in the ergot alkaloid gene cluster are being transcribed and expressed when the fungus was cultured in malt extract agar.

Figure 11: A) Mutation sites of the *P. camemberti* eas gene cluster in comparison to *P. biforme* as indicated with stars. Black stars indicate the mutations of the non coding region and are outlined in **B)**, white stars indicate the mutations within the coding regions and are further outlined in **C)**.

Figure 12: HPLC of the *P. camemberti easE* (blue) and *dmaW* (red) *N. fumigata* knockout transformants in comparison to wild type *N. fumigata* (black). This analysis indicates that the two transformants are producing fumigaclavine C (58.7), just in smaller quantities than the wild type *N. fumigata*. *EasE* transformant also appears to less efficient at converting festuclavine to fumigaclavine C, thus the larger peak at 43.8.

Figure 13: Camembert cheese prepared with *P. camemberi* NRRL 874 and *P. biforme* NRRL 885. These products were analyzed on the LC- MS for ergot alkaloids.

Figure 14: Phylogenetic analysis of the *easQ* and *easH* genes from *P. camemberti* and their relationship to those of other fungi. **A** Phylogenetic relationship between the top NCBI blastp matches of the *P. camemberti easQ* gene. It is notable that there is a paralog of the *P. camemberti* and *P. biforme easQ* gene. **B** The numerical relationships between homologues of *easQ* from other fungi with *easQ* of *P. camemberti*. **C** Phylogenetic relationship of *P. camemberti easH* and homologues from other fungi. **D** Homologues of *P. camemberti easH* based on NCBI blastp search. It is interesting to note the close relationship between *P. camemberti easH* and *Aspergillus japonicus easH*.

Figure 15: Proposed synthesis of rugulovasine from chanoclavine-I aldehyde. *EasQ*, predicted by sequence analysis to be an aldehyde dehydrogenase, would oxidize the aldehyde to an acid. *EasH*, a predicted dioxygenase, would oxidize the aromatic carbon. Dehydration would result in the formation of rugulovasine.

Figure 16: Proposed mechanisms of EasH activity as described by Jakubczyk et al. (2015) in the fungus *Aspergillus japonicus*. **A** EasH may be abstracting a hydride ion, **B** EasH may be abstracting a hydrogen ion from its substrate. **C** EasH may be hydroxylating or halogenating its substrate. Hydroxylation is the mechanism that is consistent with our hypothesis for *P. camemberti* EasH activity.

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