Ergot Alkaloids of the Family Clavicipitaceae

Simona Florea, Daniel G. Panaccione, and Christopher L. Schardl

First and third authors: Department of Plant Pathology, University of Kentucky, Lexington 40546-0312; and second author: Genetics and Developmental Biology Program, Division of Plant and Soil Sciences, West Virginia University, Morgantown 26506-6108.
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ABSTRACT

Ergot alkaloids are highly diverse in structure, exhibit diverse effects on animals, and are produced by diverse fungi in the phylum Ascomycota, including pathogens and mutualistic symbionts of plants. These mycotoxins are best known from the fungal family Clavicipitaceae and are named for the ergot fungi that, through millennia, have contaminated grains and caused mass poisonings, with effects ranging from dry gangrene to convulsions and death. However, they are also useful sources of pharmaceuticals for a variety of medical purposes. More than a half-century of research has brought us extensive knowledge of ergot-alkaloid biosynthetic pathways from common early steps to several taxon-specific branches. Furthermore, a recent flurry of genome sequencing has revealed the genomic processes underlying ergot-alkaloid diversification. In this review, we discuss the evolution of ergot-alkaloid biosynthesis genes and gene clusters, including roles of gene recruitment, duplication and neofunctionalization, as well as gene loss, in diversifying structures of clavines, lysergic acid amides, and complex ergopeptines. Also reviewed are prospects for manipulating ergot-alkaloid profiles to enhance suitability of endophytes for forage grasses.

ERGOT ALKALOIDS AND THEIR EFFECTS

Ergot alkaloids include toxins that can pose risks in food and feed but are also raw materials for therapeutic drugs to treat migraines, uterine hemorrhaging, parkinsonism, and other disorders, as well as the extremely hallucinogenic, illicit drug, lysergic acid diethylamide (LSD). These alkaloids are known from several orders of fungi in the phylum Ascomycota, and particularly plant pathogens and plant symbionts in the family Clavicipitaceae (Fig. 1). The ergot alkaloids are named for the ergot fungi, genus *Claviceps*, and are defined as derivatives of the determinant intermediate, 4-(γ,γ)-dimethylallyltryptophan (DMAT) (Fig. 2). Their biosynthetic pathways range from simple transformations of prenylated tryptophan to give tricyclic and tetracyclic forms called clavines, to incorporation—usually in the form of D-lysergic acid or D-dihydrolysergic acid—into a highly diverse array of amino-acid oligomers such as ergonovine and the ergopeptines. The latter are the most complex, diverse and dangerous ergot alkaloids, especially given the large variety of ergopeptines produced by different strains of *Claviceps purpurea* (Komarova and Tolkachev 2001). The natural ecological roles of ergot alkaloids are not well understood, though there is evidence that they can provide protection to the

producing fungi or their host plants from feeding by insects (Potter et al. 2008) and mammals (Panaccione et al. 2006b). In contrast, the pharmacological activities and pharmaceutical uses of some ergot alkaloids have been investigated extensively (van Dongen and de Groot 1995).

SOURCES OF ERGOT ALKALOIDS

Taxonomic distribution of ergot alkaloids and their biosynthesis genes. Ergot alkaloids are known from diverse members of the Pezizomycotina (filamentous ascomycetes) (Marcet-Houben and Gabaldón 2016; Wallwey et al. 2012). In addition to the family Clavicipitaceae (order Hypocreales), clavines and other ergot alkaloids have been isolated from some *Penicillium*, *Aspergillus*, and *Neosartorya* species (Trichocomaceae, Eurotiales), and from a *Dicyma* species (Xylariaceae, Xylariales). Furthermore, with increasing numbers of complete genome sequences, putative ergot-alkaloid biosynthesis (EAS) gene clusters have been identified in additional orders, such as Onygenales (*Arthroderma* and *Trichophyton* species) and Helotiales (*Glarea lozoyensis*). Given the broad distribution of ergot alkaloids and EAS genes, ergot alkaloids appear to have been an early feature in the evolution filamentous ascomycetes.

Ergot fungi. Producers of ergot alkaloids that are significant to human health and agriculture include *Claviceps* spp. (discussed here) as well as systemic symbionts (endophytes) of grasses and morning glories (discussed below). *Claviceps* spp. resting structures—called “ergots” (French for spur) or “sclerotia”—occur on florets of cereals and other grasses and can thereby contaminate grains, feed, and forage with toxic levels of ergot alkaloids or indole-diterpenes. The

*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary file is published online.

recent discovery of an apparent fossil ergot, named *Paleoclaviceps parasiticus*, indicates that ergot fungi may date to the Cretaceous (Poinar et al. 2015). Fermentation cultures of *C. purpurea* are used for industrial production of pharmaceutical ergot alkaloids (Gröger and Floss 1998).

The main danger of ergot-alkaloid poisoning in humans is by infestation of grains with ergots (sclerotia) of *Claviceps* species (Fig. 1A). The ergots are produced on infected florets of grasses (family Poaceae) or sedges (Cyperaceae) (Schumann 2000). Once its dormancy is broken, for example by overwintering in the soil, the ergot can germinate to form small mushroom-like (“stipate”) stromata with embedded, flask-shaped perithecia. The meiotically derived ascospores develop within the perithecia and are eventually ejected into the air to initiate new infections. Those that land on stigmata of their grass hosts can initiate new infection cycles (Miedaner and Geiger 2015). If the floret has already been pollinated, the stigma dries and blocks pathogen ingress. If, instead, *Claviceps* ascospores initiate infection, the fungus colonizes and overgrows the ovary, and proliferates by iterative germination to produce masses of mitotic spores (*Sphacelia*-type conidia) in a “honeydew,” which attracts insects that further spread the fungus to other florets. The fungal masses develop into ergots, thus completing the cycle.

Ergots can be very high in ergopeptines and other ergot alkaloids, as well as another class of alkaloids, the indole-diterpenes. With ergot-alkaloid concentrations in *Claviceps purpurea* ergots exceeding 1 to 2 mg/g of fungal dry mass (Pažoutová et al. 2000), these are potent sources of the mycotoxins. Adding to the risk, by virtue of their size and density, ergots mimic seeds and are difficult to remove with the chaff. Modern mechanized approaches reduce this problem, which had previously contributed to historic outbreaks of “St. Anthony’s fire,” particularly in medieval Europe (van Dongen and de Groot 1995).

Endophytic Clavicipitaceae. Many species in the family Clavicipitaceae are considered endophytes because they are systemic symbionts that persist for the lifespan of the host plant and, if vertically transmissible (seedborne), indefinitely in maternal host lineages (Bischoff and White 2003; Schardl et al. 2014a). Growth of clavicipitaceous endophytes can be mainly endobiotic or epibiotic, whereby endobiotic growth is between the plant cells within tissues, and epibiotic growth is on the surface or between, but not within host tissues. Some of these fungi are capable of vertical trans-

mission and others are not. As a prerequisite for their sexual life cycle, many produce stromata or sclerotia that replace or affect development of all or a portion of a host structure. (Like ergots, the sclerotia later give rise to stromata.) Which host structure bears the stroma or sclerotium depends on the fungal species. Often these structures occur on inflorescences or florets, but some species produce their stromata on leaves or nodes. The stromata give rise to mitotic spores and in most cases can complete the sexual cycle if opposite mating types are cross-inoculated, which for the *Epichloë* species is primarily accomplished by specialized anthomyiid flies of genus *Botanophila* (Bultman and Leuchtman 2009). There are no strict relationships between transmission mode, endobiotic versus epibiotic growth, and sexual or asexual life cycles except that strictly asexual *Epichloë* species are capable of vertical transmission (Leuchtman et al. 2014).

Ergot alkaloids are found in at least three genera of endophytic Clavicipitaceae in symbiosis with three families of plants. *Epichloë* species, which grow endobiotically and are often seed-transmitted in cool-season grasses (Poaceae, subfamily Poöideae), can be a source of various ergot alkaloids that cause important toxic syndromes in livestock (Chen et al. 2015; Klotz 2015; Shymanovich et al. 2015; Thompson and Stuedemann 1993). *Periglandula* species, which are epibiotic symbionts on buds and leaf surfaces of morning glories (Convolvulaceae, tribe Ipomoeae), produce diverse ergot alkaloids (Beaulieu et al. 2015). Other ergot-alkaloid producers include *Balansia cyperi*, an epibiont of *Cyperus* species (Cyperaceae) (Plowman et al. 1990), and *Balansia obtecta*, an endobiont of the grass *Cenchrus echinatus* (Powell et al. 1990). Neither of these *Balansia* species is seed-transmitted because they completely suppress seed production in their hosts. In the case of *Atkinsonella hypoxylon* (Kover and Clay 1998), a seed-transmissible yet pathogenic epibiont of *Danthonia* species (Poaceae), genome sequence reveals genes for the simple ergot-alkaloid chanoclavine I, which however was undetected in plants infected with this fungus (Schardl et al. 2014b).

Like *Claviceps* species, some of these endophytes produce another group of antimammalian toxins, the indole-diterpenes, and many *Epichloë* species also produce loline alkaloids and peramine, which more specifically affect invertebrates (Schardl et al. 2013c). Alkaloid profiles can differ considerably among strains within *Epichloë* species, suggesting that the sexual life cycle can be important for chemotypic

FIGURE 1

Examples of Clavicipitaceae that produce ergot alkaloids. **A**, An ergot of

Claviceps purpurea on a tall fescue inflorescence. **B**, Mycelia of *Periglandula ipomoeae* on the adaxial side of a young leaf of *Ipomoea pes-caprae*. **C**, A fertilized stroma on *Epichloë festucae* on a grass tiller.

D, Aniline blue-stained endophytic hyphae of *Epichloë coenophiala* between epidermal cells of a tall fescue leaf sheath. Arrows indicate fungal structures.

diversification (Charlton et al. 2014; Saikkonen et al. 2016; Schardl et al. 2012; Young et al. 2015). However, these studies and a survey of ergot-alkaloid profiles among *Periglandula* species (Beaulieu et al. 2015) demonstrate that quantitative and qualitative variation in alkaloid profiles can also occur within asexual endophytes.

***Epichloë* species and livestock production.** The symbiotic *Epichloë* species can confer numerous benefits to their hosts such as enhanced drought tolerance, increased competitiveness, biomass accumulation, tiller number and seed production and resistance to insects and pathogens (Malinowski and Belesky 2000; Nagabhyru et al. 2013; Schardl et al. 2004). However, when used for pasture and forage, the benefits can be significantly offset by their production of toxic alkaloids. Indole-diterpenes, such as lolitrem B, are responsible for tremors experienced by sheep and other grazing mammals (di Menna et al. 2012), whereas the ergot alkaloids cause symptoms that can vary depending the particular alkaloids and condition of the animal, such as heat or cold temperature stress (Klotz 2015; Spiers et al. 1995). Some symptoms of ergot-alkaloid toxicity in grazing livestock include elevated body temperature, reduced lactation, reduced fertility, vasoconstriction, and dry gangrene with loss of hooves (Burke et al. 2001; Gadberry et al. 2003; Klotz 2015; Thompson and Stuedemann 1993). Though they can have detrimental effects on livestock, the endophytes can be vital for the persistence of grass stands (Malinowski and Belesky 2000; Schardl et al. 2004).

Pasture and rangeland grasses with symbiotic *Epichloë* species cause symptoms characteristic either of ergot-alkaloid or indole-diterpene toxicity. The most intensively studied systems have been *Epichloë coenophiala* in tall fescue (*Lolium arundinaceum* = *Schedonorus arundinaceus*), which can cause ergot-alkaloid poisoning (Klotz 2015), and *Epichloë festucae* var. *lolii* in perennial ryegrass (*Lolium perenne*), which can cause tremors or staggers attributable to lolitrems (a group of indole-diterpenes) (di Menna et al. 2012). The latter also produce ergot alkaloids, and little consideration has been given to the possibility that they may contribute to the toxicity of endophyte-infected perennial ryegrass. Other examples of ergot-alkaloid toxicoses are reported in animals that consume drunken horse grass (*Achnatherum inebrians* with *Epichloë inebrians*) in western China (Chen et al. 2015) or sleepy grass (*Achnatherum robustum* with *Epichloë* sp.) in the southwestern United States, especially near Cloudcroft, New Mexico (Petroski et al. 1992). In contrast, indole-diterpenes probably cause the toxicoses reported in animals consuming drunkgras (*Melica decumbens* with *Epichloë melicicola*) in South Africa (Coetzer et al. 1985), or *Poa huecu* with *Epichloë tembladerae* in Argentina (Cabral et al. 1999; Pomilio et al. 1989).

BIOSYNTHETIC PATHWAYS AND VARIATIONS

Clavines and lysergic acid. Almost all steps in the ergot-alkaloid biosynthetic pathway have been elucidated (Fig. 2), and the genes involved in these steps have been identified (Table 1) (Gröger and Floss 1998; Schardl et al. 2006; Tsai et al. 1995; Wallwey and Li 2011; Young et al. 2015). These complex alkaloids vary in structure from the tricyclic clavine alkaloid, chanoclavine I, to compounds with the tetracyclic ergoline ring system found in ergopeptines and their derivatives (Fig. 2) (Schardl et al. 2006; Young et al. 2015). The ergot alkaloids are commonly categorized in four subclasses: clavines, lysergic acid, simple lysergic acid amides and ergopeptines (Correia et al. 2003; Schardl et al. 2006; Young et al. 2015).

Ergot-alkaloid biosynthesis (Fig. 2) (Gerhards et al. 2014; Robinson and Panaccione 2015; Schardl et al. 2006; Young et al. 2015) begins with the formation of 4-(γ,γ)-dimethylallyltryptophan (DMAT) from dimethylallyl diphosphate (DMAPP) and L-tryptophan, catalyzed by the enzyme, DMAT synthase (Heinstein et al. 1971), which is encoded by the gene *dmaW* (Tsai et al. 1995). The next biosynthetic step is catalyzed by DMAT *N*-methyltransferase encoded by *easF* (Otsuka et al. 1980), and subsequent steps by a

catalase encoded by *easC* (Goetz et al. 2011), and chanoclavine-I synthase, a flavine-dependent oxidoreductase encoded by *easE* (= *ccsA*) (Lorenz et al. 2010). Chanoclavine I is the simplest clavine known to be a pathway end-product, since some fungi such as *Epichloë elymi* E56 possess *dmaW*, *easF*, *easC*, and *easE* as their only four functional ergot-alkaloid biosynthesis genes (Schardl et al. 2013c). Fungal strains that produce chanoclavine I can also produce the recently discovered metabolite, ergotryptamine. The mechanism of ergotryptamine production has not yet been elucidated, but it has been shown to be a spur product rather than a direct intermediate in the pathway to chanoclavine I (Ryan et al. 2015). Genome sequencing of numerous ergot-alkaloid producers has revealed that, as expected, the genes for the four chanoclavine I production steps are present and physically linked in a cluster (the *EAS* cluster), but the vast majority of these fungi have several additional genes in their *EAS* clusters that specify elaborations to more complex ergot alkaloids (Schardl et al. 2013a, b, c, 2014a), which we now describe.

The next three catalytic steps in the pathway leading from chanoclavine I to the tetracyclic clavine alkaloid, agroclavine, are carried out in sequence by the chanoclavine I dehydrogenase encoded by *easD* (Wallwey et al. 2010), a flavine-dependent oxidoreductase ("old yellow enzyme") encoded by *easA*, and a reductase encoded by *easG* (Fig. 2). Variations in the *EasA* (Cheng et al. 2010; Coyle et al. 2010) and *EasG* (Matuschek et al. 2012) steps determine differences in the products generated. Reduction (saturation) of the C8-C9 bond is required to allow rotation around that bond, positioning the C7 aldehyde to condense with N6 to form an iminium ion. Subsequent reduction of the N6-C7 bond is catalyzed by *EasG*. In those strains of *Claviceps purpurea*, *Balansia* species, *Epichloë* species, and *Periglandula* species that produce agroclavine and its derivatives, *EasA* catalyzes reoxidation of C8-C9 after rotation. In contrast, strains of *Claviceps africana*, *Claviceps gigantea*, and *Neosartorya fumigata* produce festuclavine, which has a saturated C8-C9 bond, because their *EasA* enzymes reduce but do not reoxidize that bond (Cheng et al. 2010; Coyle et al. 2010).

Agroclavine, which is cytotoxic (Glatt et al. 1987), is transformed into various neurotropic alkaloids (Fig. 2) by the NADPH-dependent cytochrome P450 encoded by *cloA* (Fig. 2), and there is evidence that variation in *CloA* enzymes provides another source of chemotypic variation. *CloA* is apparently responsible for the three rounds of 2-electron oxidations needed to convert agroclavine to lysergic acid (Haarmann et al. 2006; Maier et al. 1988; Robinson and Panaccione 2014; Schardl et al. 2006). There is also variation in products of agroclavine oxidation, which gives lysergic acid in strains of *Claviceps purpurea*, *Balansia obtecta*, *Epichloë* species, and *Periglandula* species, but gives paspalic acid in *Claviceps paspali* (Robinson and Panaccione 2015), suggesting that the *CloA* or *EasA* isoforms specify which position in the D-ring is desaturated. *Claviceps fusiformis* PRL 1980 does not produce lysergic acid, but accumulates elymoclavine (Lorenz et al. 2007), which would require only a 2-electron oxidation of agroclavine. The enzyme(s) responsible for elymoclavine production in *Claviceps fusiformis* has not been determined.

Ergot alkaloids for which biosynthetic steps remain to be determined include lysergol and the spur products, setoclavine, isosetoclavine, and ergotryptamine (Fig. 2). Lysergol is the 9,10-desaturated isomer of elymoclavine. Setoclavine and isosetoclavine are derived from agroclavine by the action plant or fungal peroxidases (Coyle et al. 2010; Ščigelová et al. 1995). Ergotryptamine has been produced in *Emericella nidulans* (= *Aspergillus nidulans*) by heterologous expression of the genes *dmaW*, *easF*, and *easC* (Ryan et al. 2015), although the possibility remains that other *Emericella nidulans* enzymes may also be involved.

Lysergic acid amides and ergopeptines. The Clavicipitaceae exhibit extraordinary diversity of lysergic-acid derivatives (Komarova and Tolkachev 2001) generated by natural combinatorial biosynthesis involving the nonribosomal peptide synthetase (NRPS) enzymes

composed of subunits encoded by *lpsA*, *lpsB*, and *lpsC* (Ortel and Keller 2009). Most fungal NRPSs consist of single polypeptide chains that include characteristic domains arranged in several modules, whereby each module specifies a substituent that is added to the growing peptide chain, and the order of added substituents is determined by the order of modules from the N-terminus to the C-terminus of the NRPS. Lysergyl peptide synthetases (LPSs) are similar to other fungal NRPSs except that they have two subunits. LPSs catalyze biosynthesis of the ergopeptide lactams (Fig. 2), which are the immediate precursors of ergopeptines (Correia et al. 2003; Ortel and Keller 2009). One of the subunits, LpsB (=LPS2), has a single module responsible for activating the first substituent in the chain, D-lysergic acid (or D-dihydrolysergic acid), whereas LpsA (=LPS1) has three modules that each specify a hydrophobic L-amino acid such as those commonly found in proteins (Ala, Ile, Leu, Met, Phe, Pro, or Val), or an analog such as L-homoalanine (2-aminobutyric acid), L-norleucine (2-aminohexanoic acid), or L-homoisoleucine (2-amino,3-methylhexanoic acid) (Fig. 3). Once these four substituents are linked, the product is released from the

NRPS while cyclizing the second and third amino acid to form the piperizinedione ring of the ergopeptide lactam. Finally, oxidative cyclization catalyzed by the dioxygenase, EasH, generates the cyclol ring of the completed ergopeptine (Havemann et al. 2014).

An advantage of the two-subunit arrangement of the LPS is that one subunit need not be faithful to the other, and in several Clavicipitaceae LpsB can alternatively complex with LpsC (=LPS3) to form the enzyme responsible for ergonovine biosynthesis (Ortel and Keller 2009). The LpsC subunit contains a single module of the canonical NRPS type, plus a C-terminal thioreductase domain. Thus, the LpsB/LpsC complex catalyzes a peptide linkage of D-lysergic acid to Ala, followed by reductive release from the enzyme cofactor to give ergonovine.

In *Epichloë inebrians* and *Periglandula ipomoeae* two additional genes may be required to produce lysergic acid α -hydroxyethylamide (LAH): *easO*, encoding a putative oxygenase, and *easP*, encoding a putative LAH synthase (Scharidl et al. 2013b). In turn LAH can spontaneously degrade to release ergine (lysergic acid amide) (Flieger et al. 1982). Although this is not the only path to

TABLE 1
Ergot-alkaloid biosynthesis gene names and encoded enzyme functions^a

Gene name	Function	Genes and synonyms		
		<i>Neosartorya fumigata</i> Af293	<i>Periglandula ipomoeae</i> lasaF13	<i>Claviceps purpurea</i> 20.1
<i>dmaW</i>	4-(γ,γ)-dimethylallyltryptophan synthase	<i>fgaPT2</i>	<i>dmaW</i>	<i>Cpd1</i>
<i>easF</i>	4-(γ,γ)-dimethylallyltryptophan <i>N</i> -methyltransferase	<i>fgaMT</i>	<i>easF</i>	<i>easF</i>
<i>easC</i>	Catalase involved in chanoclavine I synthesis	<i>fgaCat</i>	<i>easC</i>	<i>easC</i>
<i>easE</i>	Chanoclavine I synthase	<i>fgaOx1</i>	<i>easE</i>	<i>ccsA</i>
<i>easD</i>	Chanoclavine I dehydrogenase	<i>fgaDH</i>	<i>easD</i>	<i>easD</i>
<i>easA</i>	Chanoclavine-I aldehyde oxidoreductase; old yellow enzyme; reduction only (reductase), or followed by oxidation (isomerase), of C8 = C9 bond	<i>fgaOx3</i>	<i>easA</i>	<i>easA</i>
<i>easG</i>	Agroclavine, festuclavine or pyroclavine synthase; reduction of C7 = N6 iminium ion.	<i>fgaFS</i>	<i>easG</i>	<i>easG</i>
<i>easK</i>	Cytochrome P450; unknown role	<i>fgaP450-1</i>	–	–
<i>easL</i>	Fumigaclavine reverse prenyltransferase	<i>fgaPT1</i>	–	–
<i>easM</i>	Cytochrome P450, festuclavine 9-monooxygenase	<i>fgaP450-2</i>	–	–
<i>easN</i>	Fumigaclavine acetylase	<i>fgaAT</i>	–	–
<i>cloA</i>	Agroclavine, festuclavine, or elymoclavine monooxygenase, oxidation of C17 to alcohol or carboxyl	–	<i>cloA</i>	<i>cloA</i>
<i>lpsB</i>	Lysergyl peptide synthetase subunit 2, specifying lysergic acid	–	<i>lpsB</i>	<i>Cpps2</i>
<i>lpsA</i>	Lysergyl peptide synthetase subunit 1, specifying amino acids 1, 2 and 3	–	<i>lpsA</i>	–
<i>lpsA1</i>	Lysergyl peptide synthetase subunit 1, for ergotamam biosynthesis	–	–	<i>Cpps1</i>
<i>lpsA2</i>	Lysergyl peptide synthetase subunit 1, for ergocryptam biosynthesis	–	–	<i>Cpps4</i>
<i>lpsC</i>	Lysergyl peptide synthetase and reductase subunit, specifying L-alanine	–	<i>lpsC</i>	<i>Cpps3</i>
<i>easO</i>	FAD-containing monooxygenase; putative ergonovine dehydrogenase	–	<i>easO</i>	–
<i>easP</i>	Alpha/beta hydrolase fold protein; putative lysergic acid α -hydroxyethylamide (LAH) synthase	–	<i>easP</i>	–
<i>easH</i>	Cycloclavine synthesis (<i>Aspergillus japonicus</i>), or ergopeptide lactam hydroxylase (Clavicipitaceae)	<i>easH^ψ</i>	<i>easH</i>	<i>easH</i>

^a For genes named by the *abcD* convention, the encoded protein can be designated in the form AbcD or based on its enzyme activity. Duplicated genes are designated in the forms *abcD1* and *abcD2*. Superscripted psi (ψ) indicates a pseudogene.

ergine, which can be generated in small amounts in a pathway that requires LpsA (Panaccione et al. 2003), LAH producers generally produce ergine as well (Beaulieu et al. 2015; Robinson and Panaccione 2015).

Although ergopeptines are the largest, most complex forms of ergot alkaloids, they do not necessarily represent evolutionary endpoints. Comparative genomics and chemotypic profiling of Clavicipitaceae—especially species and strains of *Epichloë*—indicate dynamic evolution sometimes toward simpler forms or even complete loss of these alkaloids, as discussed in the next section.

EVOLUTION OF ERGOT-ALKALOID BIOSYNTHETIC PATHWAYS

Metabolic “inventions.” Here we present a hypothesis for evolution of the pathway and chemotypic diversity of the Clavicipitaceae, which we base on current knowledge of the enzymes and genes, and comparative genomics of seven genera of ergot-alkaloid producers. In Figure 4 we map evolutionary events, including what we call “inventions” of ergot-alkaloid subclasses onto a phylogeny inferred from aligned concatenated sequences of the four early pathway genes. One of these inventions, the fumigaclavine group of alkaloids, maps to the Trichocomaceae branch (represented in Figure 4 by *N. fumigata*), whereas other inventions are associated with Clavicipitaceae. The first branch to characteristic alkaloids of the Clavicipitaceae involves invention of lysergic acid and its complex derivatives, the ergopeptines. After this, as evidenced by comparing *EAS* and housekeeping gene phylogenies (Young et al. 2015), an apparent duplication of the entire *EAS* cluster gave two paralogous clades, of which one now comprises the genes from most *Epichloë* spp., and the other comprises the *EAS* genes in other genera of Clavicipitaceae plus *Epichloë inebrians*. Apparently the *EAS* clusters of the main *Epichloë*-associated clade have remained subterminal (closely linked to a telomere), whereas those in most other Clavicipitaceae are located internally on chromosomes. On the branch to the latter clade we map invention of the ergonovine-LAH-ergine pathway. Then, within the *Claviceps* subclade, we map invention of the dihydroergot alkaloids, which can be important sources of pharmaceuticals that have fewer side effects than the ergopeptines (de Groot et al. 1998).

Multiple gene neofunctionalizations and gene losses have led to the various metabolic inventions and further diversification of ergot-alkaloid structures. Neofunctionalization of a biosynthetic enzyme involves changes in substrate specificity, metabolic product or both, and is associated with gene recruitment, divergence or duplication. Gene losses also help drive diversification, particularly for a complex biosynthetic pathway or network such as ergot-alkaloid biosynthesis. Interestingly, whereas gene losses seem very common, the phylogeny (Fig. 4) is consistent with the possibility that new genes were recruited to *EAS* clusters mainly or exclusively when those clusters were subterminal. The possibility that a subterminal location facilitates the process of gene recruitment would help to explain why specialized (secondary) metabolism (SM) gene clusters are often subterminal.

Mapping neofunctionalization events parsimoniously on the *EAS* gene phylogeny (Fig. 4) provides a hypothesis as to how these processes have contributed to the evolution of ergot-alkaloid diversity. Genes *dmaW*, *easF*, *easC*, and *easE* are common to all ergot-alkaloid producers, and determine the first four steps of the pathway. As with all *EAS* genes, there are homologs for other metabolic processes, so it seems reasonable to assume that these were recruited and neofunctionalized from genes for steps in other pathways. Neofunctionalization would similarly have given rise to the *easD*, *easA*, and *easG* genes for the next three steps that yield agroclavine or festuclavine.

Some of these early recruited genes have different activities in different taxa. For example, in most Clavicipitaceae EasA functions to reduce and later reoxidize (thereby isomerize) the C8–C9 bond, whereas in the Trichocomaceae, as well as in *Claviceps africana*

and *Claviceps gigantea*, EasA only catalyzes reduction of this bond (Coyle et al. 2010). The phylogeny does not indicate which is the ancestral activity. Trichocomaceae and Clavicipitaceae also have homologous *easH* genes, but with very different functions. The EasH isoform in *Aspergillus japonicus* is involved in cycloclavine production (Jakubczyk et al. 2015), whereas the isoforms in Clavicipitaceae catalyze the last step in ergopeptine biosynthesis (Havemann et al. 2014).

Like the common *EAS* genes, those encoding pathway-specific enzymes would have been recruited and neofunctionalized, giving either festuclavine and fumigaclavines typical of the Trichocomaceae (genes *easM*, *easN*, and *easL*), or the clavines, lysergic acid and ergopeptines typical of the Clavicipitaceae (genes *cloA*, *lpsA*, and *lpsB*). In Figure 4 we map these gene recruitments after divergence of the two families, but it is also possible that some of these genes were present in the ancestral gene cluster and then lost in evolution of one or the other fungal family.

Diversification of ergopeptines is an excellent example of gene duplication and neofunctionalization. After the *EAS* cluster duplicated, substrate specificities of the second and third modules of the NRPS, LpsA, evolved such that *Epichloë* species now produce mainly ergovaline (Lyons et al. 1986), and *Balsania* and *Periglandula* species now produce mainly ergobalansine (Beaulieu et al. 2015; Powell et al. 1990). (Whichever was the ancestral ergopeptine cannot be discerned from the available data.) Remarkably, LpsA neofunctionalization appears to have accelerated dramatically in the *Claviceps* clade to give vast majority of known ergopeptines shown in Figure 3 (Blaney et al. 2003; Cvak et al. 1994, 1996, 2005; Eleuterius and Meyers 1977; Komarova and Tolkachev 2001; and references therein; Perellino et al. 1993).

Further NRPS evolution was key to a separate branch of the pathway. The first module of *lpsA* appears to have been duplicated and this single-module derivative was fused to a recruited thioreductase-coding domain at its 3' end. Thus evolved LpsC (Ortel and Keller 2009), which retained properties of the first LpsA module; namely, its ability to complex with LpsB and its specificity for L-alanine (Ala). The two-subunit LPS composed of LpsB and LpsC links lysergic acid to Ala, and the product is reductively released from the enzyme-bound pantothenic acid to give ergonovine. Interestingly, on that same branch of the phylogeny we map the origin of *easO* and *easP*, presumed to specify conversion of ergonovine to LAH, an unstable product that spontaneously hydrolyses to the simple lysergic acid amide, ergine. As with other new genes, it seems likely that *easO* and *easP* were recruited and neofunctionalized from genes for other metabolic pathways.

All considered, invention of the ergonovine/LAH/ergine pathway (*lpsC*, and hypothetically *easO* and *easP*) represents a remarkably complex set of evolutionary events that leads to much simpler amides of lysergic acid compared with the acutely toxic ergopeptines. This seems to contradict the common paradigm that systems evolve toward greater complexity, but these simpler lysergic acid amides may have been selectively favored in some systems because they are less costly for the fungus to produce. Although there is overlap of symptoms associated with simpler and complex lysergic acid amides, mammalian grazers that ingest ergopeptines can exhibit acute toxicoses, whereas those ingesting the simpler lysergic acid amides present in sleepy grass (*Achnatherum robustum*) and drunken horse grass (*Achnatherum inebrians*) exhibit inebriation and are deterred from future grazing of these plants (Chen et al. 2015; Shymanovich et al. 2015).

Gene loss as a diversifying mechanism. More recent chemotypic diversification between members of closely related genera or species, or even different strains of a species, have primarily involved gene losses, although neofunctionalizations of existing *EAS* cluster genes also are evident. Often strains that have lost genes retain partial gene sequences (remnants), further supporting the scenario that their ancestors had more complete *EAS* clusters. Gene losses could eliminate either of the two branches to ergopeptines or

A *Continued on next page***FIGURE 3**

Ergopeptines. **A**, Ergopeptines with aa2 = valine, phenylalanine, methionine, isoleucine, homoisoleucine, or homoalanine. Only the ergopeptines in square brackets (β-ergosine and β-ergoptine) remain unknown as natural compounds. **B**, Ergopeptines with aa2 = norleucine or leucine. Amino acid precursors are indicated as aa1, aa2, and aa3 in the order that they are added by the LpsA subunit of lysergyl peptide synthetase (LPS). Note the variation in aa3, and the dihydrolysergic acid moiety of dihydroergosine.

simple lysergyl amides, or they could prune the pathway back all the way to a tetracyclic clavine (e.g., elymoclavine, as in *Claviceps fusiformis*) or the tricyclic chanoclavine I. Interestingly, the natural evolutionary processes are usually more complex than necessary for

the chemotypic change. An example is the evolution from ancestors that produce ergopeptines to strains that terminate the pathway at chanoclavine I. Although this only requires loss of *easD*, both occurrences—to an *Epichloë* clade and to *Atkinsonella hypoxylon*—have

FIGURE 3

Continued from previous page.

Phylogeny of *dmaW* + *easF* + *easC* + *easE* CDS

Alkaloids

FIGURE 4

Evolutionary hypothesis for ergot-alkaloid diversification. Evolutionary events are mapped onto a phylogeny based on concatenated coding sequences (CDS) for *dmaW*, *easF*, *easC*, and *easE* (Supplementary File), annotated from whole genome sequences, aligned by MUSCLE (Edgar 2004), and inferred by maximum likelihood implemented in PhyML (Dereeper et al. 2008). Internal branches drawn with thick lines received ≥ 0.99 ALR support (Anisimova and Gascuel 2006). Evolutionary inventions of major groups of ergot alkaloids are indicated in yellow boxes, gene neofunctionalizations are indicated in red boxes, shifts between subterminal locations (near telomeres) and internal locations are indicated in blue boxes, and gene recruitments and losses are indicated in unshaded boxes. Asterisks (*) indicate the presence of gene remnants. Alkaloids listed at right are confirmed (bold text), predicted but not tested on the strains indicated (regular type), or predicted from gene contents but undetected (in parentheses).

involved losses of all six downstream genes (Fig. 4). This is a common phenomenon, such that we can identify seven evolutionary branches with multiple gene losses, and in only one case did a possibly functional gene remain for a downstream step (*easH* in *Claviceps fusiformis*). This tendency for multiple gene loss suggests consistent selection against genes for enzymes that no longer have a substrate.

We can speculate on the selective basis for frequent losses of multiple genes, rather than single genes. Once an enzyme has become useless due to loss of an upstream gene, perhaps continued production of the enzyme exacts a significant cost in metabolic resources. Another intriguing possibility is that, when they lack their normal substrates, downstream enzymes will catalyze other reactions, of which some will convert cellular metabolites to detrimental products. That raises yet another possibility, that some such enzymes could act on other SM or their intermediates to give products with improved protective or otherwise beneficial characteristics. An empirically supported model for SM enzyme evolution holds that they tend to be less substrate specific than primary metabolic enzymes (Firn and Jones 2000). Thus, the respective gene could be recruited for the other SM pathway, which might explain the tendency for different SM pathways to share a hodgepodge of enzyme homologs that have been repurposed (neofunctionalized) for those pathways.

ERGOT-ALKALOID DIVERSIFICATION IN THE CLAVICIPITACEAE

Variations in *EAS* clusters. The presence or absence of genes in the *EAS* cluster(s) determines the ergot-alkaloid profile of the fungus (Schardl et al. 2013c). So far, 14 genes—*dmaW*, *easF*, *easC*, *easE*, *easD*, *easA*, *easG*, *cloA*, *lpsB*, *lpsA*, *lpsC*, *easH*, *easP*, and *easO*—have been shown or proposed to direct steps in the ergot-alkaloid pathways of Clavicipitaceae (Table 1), and only *Periglandula ipomoeae* is known to harbor all of them (Young et al. 2015). The state of research to date is such that enzyme activities have been documented for the products of 12 of the 14 genes, with only *easP* and *easO* remaining with unconfirmed functions. In the previous section we described the pathway, and identified some points of divergence where variation in the enzyme isoform results in product differences. Such divergence points are specified by *easA*, *easG*, *lpsA*, and perhaps *cloA*.

The typical ergot-alkaloid profile of plants with *Periglandula* species includes some or all of chanoclavine I, ergobalansine, ergonovine, lysergic acid α -hydroxyethylamide (LAH) and ergine, in addition to which low levels of lysergol or high levels of cycloclavine have been observed in some plants (Beaulieu et al. 2015). Ergot fungi (*Claviceps* species) produce an even wider spectrum of ergot alkaloids. Strains of *Claviceps purpurea* are capable of producing ergonovine and the complex ergopeptines because their *EAS* clusters include all known ergot-alkaloid genes except *easO* and *easP* (Negård et al. 2015; Schardl et al. 2013c). In addition, *Claviceps purpurea* strain 20.1 has two very similar *lpsA* genes (*lpsA1* and *lpsA2*), due to tandem duplication and neofunctionalization, to give ergotamine and ergocryptine as its two main ergopeptines (Haarmann et al. 2008). On the other hand, the *EasA* isoform in *Claviceps africana* directs the pathway toward festuclavine and dihydroergosine. *Claviceps fusiformis* has lost genes for late steps, so it produces elymoclavine, but no ergopeptines (Lorenz et al. 2007).

Pathway control. The *Epichloë* species generally produce their alkaloids only in symbio (Chujo and Scott 2014), and host genotype can influence the alkaloid levels (Takach et al. 2012). Alkaloid levels can also be altered by modifying gene expression levels. In RNA interference (RNAi) experiments with the perennial ryegrass endophyte, *Epichloë festucae* var. *lolii* $\times$ *Epichloë typhina* Lp1, levels of chanoclavine I and ergotryptamine were significantly elevated, and levels of ergovaline declined, concomitant with reduction of *easA* mRNA (Mulinti et al. 2016). Overexpression of

easA had the opposite effect. These results demonstrate quantitative genetic control of ergot-alkaloid profiles.

In addition to the pathway end-products (Fig. 2), fungi typically accumulate substantial amounts of intermediates and spur products. For example, in some strains, such as *Epichloë elymi* E56, the pathway ends with chanoclavine I (Schardl et al. 2013c), but in toxic strains of *Epichloë coenophiala* this is an early intermediate in the ergovaline pathway. Nevertheless, chanoclavine I is typically present in plants with *Epichloë coenophiala* or other ergovaline producers, often at levels comparable to, or exceeding, those of ergovaline (Florea et al. 2016; Mulinti et al. 2016). Such pathway inefficiencies have been observed in several *Epichloë*, *Claviceps*, and *Periglandula* species, as well as in *N. fumigata*, and suggest a selective benefit of alkaloid diversity within the individual fungus or its host plant (Beaulieu et al. 2015; Panaccione 2005).

No pathway-specific regulatory gene has been identified in the vicinity of *EAS* clusters, but telomere localization in many genera, and histone modifications (“marks”) may be key to epigenetic control. In *Epichloë festucae*, Chujo and Scott (2014) deleted the gene, *clrD*, encoding histone H3K9 methyltransferase, and the gene, *ezhB*, encoding histone H3K27 methyltransferase. Both mutations reduced the corresponding histone marks globally and in alkaloid gene clusters, and derepressed several genes in the ergot alkaloid and indole-diterpene gene clusters. When two independent *ezhB*-deletion mutants were introduced into perennial ryegrass, the plants accumulated several-fold higher levels of chanoclavine I and ergovaline relative to plants with the wild-type endophyte. Interestingly, relative amounts of the intermediate, chanoclavine I, and the end-product, ergovaline, remained relatively similar even with much higher accumulation of both products. The basis for the inherent inefficiency of the pathway, and possible selective advantages for the resulting diversity of alkaloids in each endophyte-symbiotic plant, are intriguing subjects for future research.

Why clusters? Not all assemblies of sequenced Clavicipitaceae genomes have brought together entire *EAS* gene clusters, but single contiguous clusters are known in *Claviceps fusiformis*, *Claviceps purpurea*, *Epichloë coenophiala*, *Epichloë festucae*, and *Periglandula ipomoeae*, as well as in *N. fumigata* (Florea et al. 2016; Schardl et al. 2013c; Young et al. 2015). Only *Epichloë inebrians* unambiguously possesses separate clusters with different sets of *EAS* genes, and the introduction of a telomere (which is just 1 bp downstream of the *cloA* stop codon) may have been responsible for this arrangement (Fig. 5). The tendency of genes to cluster is also apparent for other alkaloid classes (Schardl et al. 2013c), and is a well known characteristic of fungal SM genes (Spatafora and Bushley 2015).

A few ideas have been floated regarding selective forces for SM-genes clustering. One is that clustered genes may express coordinately. Results of the recent epigenetic study of Chujo and Scott (2014) are not fully supportive of this hypothesis because deletion of histone demethylation enzymes generally caused changes of gene expression in some but not all of the genes in each cluster. Furthermore, it is certainly not necessary for organisms to cluster genes in order to coordinately regulate them.

Another set of hypotheses has to do with coinheritance of entire gene clusters (Walton 2000). It is difficult to envisage how horizontal (lateral) gene transfers (HGT) between distantly related fungi could provide the selective basis for clustering. Such a process has not been observed directly in fungi, and if it happens at all it is probably far too infrequent to be the primary process selecting for gene clustering. In that regard, a recent claim that *EAS* genes were horizontally transferred to Clavicipitaceae (Marcet-Houben and Gabaldón 2016) is dubious because it includes gene orthologs and paralogues in the phylogenetic analysis (a typical problem in HGT studies), and because the purported discontinuous distribution among fungal groups is overstated. Though best characterized in Clavicipitaceae and Trichocomaceae, an ergopeptine has been isolated from a *Dicyma* species (Xylariaceae) (Vazquez et al. 2003),

and ergot-alkaloid biosynthesis genes or close homologs have been identified in diverse fungi (Gerhards et al. 2015) including a *Malbranchea* sp. (Myxotrichaceae) (Ding et al. 2008).

We suggest that SM gene clusters evolve in the context of sexually recombining genomes. Considering that presence/absence polymorphisms are very common, sexual recombination could easily generate strains with only partial sets of the genes necessary for a finished metabolite, especially if those genes are not clustered. As discussed above, strains lacking a determinant gene for a branch in the pathway have generally lost the whole consortium of genes for that branch. This observation strongly suggests selection against genes for enzymes that lack their normal substrates, an apparently disfavored situation that would be far more likely to arise from sexual recombination if the genes are dispersed in the genome rather than being clustered.

MANIPULATION OF ERGOT-ALKALOID PROFILES

Species of *Lolium*, *Schedonorus*, and *Festuca* are among the most popular grasses for forage and pasture, and their interactions with endophytic *Epichloë* species have been well studied. The endophytes provide agroecological benefits to their hosts, and their presence can be crucial for longevity of the grass stand (Hopkins et al. 2010). The endophytes induce beneficial changes in host growth, physiological competitiveness and drought tolerance (Li et al. 2014; Nagabhyru et al. 2013; Schardl et al. 2004). However, *Epichloë* species are best known for their production of anti-herbivore alkaloids, of which the aminopyrrolizidines (lolines) and a pyrrolopyrazine (peramine) are desirable for protection from invertebrates, whereas ergot alkaloids and indole-diterpenes can be toxic to livestock (Schardl et al. 2013a).

Periglandula ipomoeae IasaF13

Claviceps purpurea 20.1

Claviceps fusiformis PRL 1980

Epichloë inebrians e818

Epichloë festucae Fl1

Epichloë elymi E56

Epichloë coenophiala e4163

Neosartorya fumigata Af293

FIGURE 5

Maps of ergot-alkaloid biosynthesis (*EAS*) gene clusters in some Clavicipitaceae and *Neosartorya fumigata* (Trichocomaceae). Black box-arrows indicate *EAS* genes, which are labeled with the full name or an abbreviation with the last letter of the gene name, or with *B* for *cloA*, followed by a numeral for those with multiple copies in the respective genome. White boxes indicate pseudogenes (designated with a superscript Ψ), and gray boxes indicate genes for functions other than ergot-alkaloid biosynthesis. Breaks in horizontal lines on the maps indicate breaks in the genome sequence assemblies, where linkages and gap lengths are currently unknown. End-product ergot alkaloids are labeled by species name in Figure 2.

Technologies for genetic manipulations of fungal endophytes have been developed and used to elucidate the roles and regulation of *EAS* genes, as well as to reduce toxicity of endophytes for grass cultivars (Florea et al. 2016; Mulinti et al. 2016; Robinson and Panaccione 2015; Wang et al. 2004). Also, such technologies could lead to identification of compounds with useful pharmaceutical properties.

To demonstrate their roles in the ergot-alkaloid pathway, and to generate biological material to test ergot-alkaloid effects on insects, nematodes and mammals, the genes *dmaW* (Wang et al. 2004) and *lpsA* (Panaccione et al. 2001) were inactivated in a perennial ryegrass endophyte, *Epichloë festucae* × *typhina* Lp1. An engineered partial deletion of *dmaW* (Δ *dmaW*) failed to produce ergot alkaloids, confirming that DMAT synthase is the determinant step of the pathway. Panaccione et al. (2001) disrupted *lpsA* by transformation and homologous recombination in Lp1, and the alkaloid profile of the resulting Δ *lpsA* strains contained clavines and lysergic acid, but none of the products downstream from lysergic acid such as ergovaline and ergine. Food preference and satiety experiments were performed on rabbits using plants with Δ *dmaW*, Δ *lpsA*, or wild-type Lp1, or plants that were endophyte-free. Plants containing the Δ *dmaW* ergot alkaloid-free strains were preferred by rabbits over plants with Δ *lpsA*, which produce clavine alkaloids but not ergovaline, suggesting that clavine alkaloids were sufficient to deter rabbit herbivory (Panaccione et al. 2006b). Tests employing the same material provided to black cutworm (*Agrotis ipsilon*) indicated that ergovaline was the principle anti-insect ergot alkaloid, and that clavines had little effect on this insect (Potter et al. 2008).

Wild-type Lp1 also significantly suppressed reproduction of the root-associated endoparasitic nematode, *Pratylenchus scribneri*, but this effect was similar for Δ *dmaW* or Δ *lpsA* endophytes, indicating that ergot alkaloids were not involved (Panaccione et al. 2006a). These results are consistent with the distribution of ergot alkaloids, estimated to be approximately 100-fold higher in shoots than roots (Malinowski et al. 1998). It would be interesting to determine if ergot alkaloids affect foliar nematodes such as *Anguina* spp., which vector the corynetoxin-producing bacterium, *Rathayibacter toxicus*. If so, ergot alkaloids may help prevent annual ryegrass toxicity (McKay and Ophel 1993; Riley et al. 2003).

Most common tall fescue cultivars have endophyte strains that produce ergovaline, which is considered the main agent in fescue toxicosis suffered by grazing livestock (Klotz 2015). Researchers from New Zealand and Arkansas have surveyed Mediterranean ecotypes to find suitable tall fescue endophytes that lack ergot alkaloids. These have been introduced into tall fescue lines to produce cultivars that are nontoxic to livestock, which have become popular in U.S. markets (Bouton et al. 2002; Gunter and Beck 2004; Hopkins et al. 2010). However, perhaps because they derive from different ecotypes, these endophytes do not necessarily provide all plant performance enhancements of the toxic, 'Kentucky 31'-derived endophytes, particularly with respect to antinematode activities (Timper et al. 2005). For this reason, surgical elimination of key ergot-alkaloid genes from 'Kentucky 31' endophytes is underway (Florea et al. 2009, 2016).

Molecular genetic strategies to generate *Epichloë coenophiala* strains that are nontoxic but also marker-free are practical because this endophyte only transmits through the seeds of tall fescue, and any such novel endophyte remains strictly associated with the cultivars in which it is introduced. This approach has its difficulties, however, because the fungus grows slowly in culture, exhibits low frequencies of homologous recombination following transformation, and possesses two paralogous *EAS* clusters (*EAS1* and *EAS2*) (Florea et al. 2016). Two strategies were applied to *Epichloë coenophiala* strain e19 to eliminate key genes in each of its two *EAS* clusters. The first strategy was marker-exchange mutagenesis of *dmaW2*, which was performed with a hygromycin-resistance gene marker (*hph*) flanked by loxP sites. Subsequently, transient ex-

pression of Cre enzyme causes recombination of the loxP-sites, eliminating *hph* to generate a marker-free Δ *dmaW2* mutant (Florea et al. 2009).

The second approach took advantage of the subterminal location of *Epichloë coenophiala* *EAS1*, and employed a novel strategy to eliminate the chromosome end in a way that destabilized the introduced DNA. *EAS1* was thereby removed with no net introduction of foreign genes (Florea et al. 2016). Applied to wild-type *Epichloë coenophiala* e19, the method generated a Δ *EAS1* mutant that retained early pathway steps, and applied to a Δ *dmaW2* mutant, the method generated a Δ *dmaW2* Δ *EAS1* strain that lacked both copies of the gene for the first determinant step of the pathway.

The mutated strains were introduced into tall fescue to evaluate their alkaloid profiles (Florea et al. 2016). The Δ *dmaW2* Δ *EAS1* strain lacked ergot alkaloids, and the Δ *EAS1* strain lacked ergovaline, but produced chanoclavine I and ergotryptamine. Genome sequence results suggested that the Δ *EAS1* chemotype was due to a defect in *lpsB2*, which was confirmed by complementation with a functional *lpsB* gene (Florea et al. 2016). It is unknown why the Δ *EAS1* strain did not accumulate lysergic acid, but unpublished results so far suggest that the reason involves regulation of gene expression, enzyme activities or both.

The obvious next steps, once any regulatory concerns are addressed, are to evaluate tall fescue with Δ *EAS1* and Δ *dmaW2* Δ *EAS1* strains, to test effects on agronomic characteristics of the grass as well as the health of grazing livestock.

FUTURE PROSPECTS

The ergot alkaloids and their medical, agricultural and ecological importance will remain fertile grounds for research in the foreseeable future. Despite the sordid history of LSD, ergot alkaloids continue to expand their importance in medicine, such as in their recent deployment in treatment of parkinsonism. Meanwhile, efforts will continue toward prevention or amelioration of ergot-alkaloid toxicity in livestock that receive exposure from ergots (*Claviceps* spp.) or grasses with related endophytes (*Epichloë* spp. and others), while the ecological roles and evolutionary selection for ergot-alkaloid diversity in Clavicipitaceae, Trichocomaceae, and other fungi deserve much more research than they have had to date. Both agricultural and ecological research stand to gain with the identification (Marlatt et al. 1997), as well as generation by targeted genetic modifications (Florea et al. 2009; Florea et al. 2016), of chemotypic variants with or without ergot alkaloids, or with different clavine and ergot-alkaloid profiles. Furthermore, novel approaches to generate nontoxic endophytes of forage grasses should help to tailor forage cultivars for livestock and equine husbandry.

The ergot-alkaloid pathway is remarkably well characterized, with just a few genes remaining for functional demonstration (*easK*, *easO*, and *easP*), and just a few major products for which biosynthetic mechanisms are mostly obscure (e.g., ergotryptamine, lysergol, LAH, and ergine). Nevertheless, as researchers further elucidate activities of enzymes encoded by the *EAS* genes, numerous intriguing questions arise regarding their structure-activity relationships and how they have evolved and neofunctionalized. While it was long suspected, and then confirmed (Haarmann et al. 2008), that variations in *LpsA* modules determine the different ergopeptines, recent studies have indicated at least four other enzymes with different activities in different systems: *EasA* variants either isomerize or reduce chanoclavine I aldehyde (Coyle et al. 2010), *EasG* variants specify festuclavine or its stereoisomer, pyroclavine (Matuschek et al. 2012), *CloA* variants catalyze 2-electron or 6-electron oxidations of agroclavine, respectively, to elymoclavine or lysergic acid (or its isomer, paspalic acid), and very different substrate specificities of *EasH* variants catalyze important oxidations in the synthesis of cycloclavine or ergopeptines (Havemann et al. 2014; Jakubczyk et al. 2015). Thus, from the first demonstration that the determinant

enzyme for the ergot-alkaloid pathway belonged to a previously unknown family of prenyltransferases (Metzger et al. 2009; Tsai et al. 1995), this pathway has been a treasure trove for investigations of various enzyme families including catalases, flavin-containing “old yellow enzymes,” dehydrogenases, cytochromes P450, nonribosomal peptide synthetases, nonheme iron dioxygenases and more.

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LITERATURE CITED

- Anisimova, M., and Gascuel, O. 2006. Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. *Syst. Biol.* 55:539-552.
- Beaulieu, W. T., Panaccione, D. G., Ryan, K. L., Kaonongbua, W., and Clay, K. 2015. Phylogenetic and chemotypic diversity of *Periglandula* species in eight new morning glory hosts (Convolvulaceae). *Mycologia* 107:667-678.
- Bischoff, J. F., and White, J. F., Jr. 2003. The plant-infecting clavicipitaleans. Pages 125-149 in: *Clavicipitalean Fungi: Evolutionary Biology, Chemistry, Biocontrol and Cultural Impacts*. J. F. White Jr., C. W. Bacon, N. L. Hywel Jones, and J. W. Spatafora, eds. Marcel-Dekker, Inc., New York, Basel.
- Blaney, B. J., Maryam, R., Murray, S.-A., and Ryley, M. J. 2003. Alkaloids of the sorghum ergot pathogen (*Claviceps africana*): Assay methods for grain and feed and variation between sclerotia/sphacelia. *Aust. J. Agric. Res.* 54:167-175.
- Bouton, J. H., Latch, G. C. M., Hill, N. S., Hoveland, C. S., McCann, M. A., Watson, R. H., Parish, J. A., Hawkins, L. L., and Thompson, F. N. 2002. Reinfection of tall fescue cultivars with non-ergot alkaloid-producing endophytes. *Agron. J.* 94:567-574.
- Bultman, T. L., and Leuchtmann, A. 2009. Biology of the *Epichloë-Botanophila* interaction: An intriguing association between fungi and insects. *Fungal Biol. Rev.* 22:131-138.
- Burke, J. M., Spiers, D. E., Kojima, F. N., Perry, G. A., Salfen, B. E., Wood, S. L., Patterson, D. J., Smith, M. F., Lucy, M. C., Jackson, W. G., and Piper, E. L. 2001. Interaction of endophyte-infected fescue and heat stress on ovarian function in the beef heifer. *Biol. Reprod.* 65:260-268.
- Cabral, D., Cafaro, M. J., Saidman, B., Lugo, M., Reddy, P. V., and White, J. F., Jr. 1999. Evidence supporting the occurrence of a new species of endophyte in some South American grasses. *Mycologia* 91:315-325.
- Charlton, N. D., Craven, K. D., Afkhami, M. E., Hall, B. A., Ghimire, S. R., and Young, C. A. 2014. Interspecific hybridization and bioactive alkaloid variation increases diversity in endophytic *Epichloë* species of *Bromus laevipes*. *FEMS Microbiol. Ecol.* 90:276-289.
- Chen, L., Li, X. Z., Li, C. J., Swoboda, G. A., Young, C. A., Sugawara, K., Leuchtmann, A., and Schardl, C. L. 2015. Two distinct *Epichloë* species symbiotic with *Achnatherum inebrians*, drunken horse grass. *Mycologia* 107:863-873.
- Cheng, J. Z., Coyle, C. M., Panaccione, D. G., and O'Connor, S. E. 2010. Controlling a structural branch point in ergot alkaloid biosynthesis. *J. Am. Chem. Soc.* 132:12835-12837.
- Chujo, T., and Scott, B. 2014. Histone H3K9 and H3K27 methylation regulates fungal alkaloid biosynthesis in a fungal endophyte-plant symbiosis. *Mol. Microbiol.* 92:413-434.
- Coetzer, J. A. W., Kellerman, T. S., and Naude, T. W. 1985. Neurotoxicoses of livestock caused by plants and fungi in southern Africa. Report No. 199. Page 38. Technical Communication, Department of Agriculture, South Africa, Onderstepoort, South Africa.
- Correia, T., Grammel, N., Ortel, I., Keller, U., and Tudzynski, P. 2003. Molecular cloning and analysis of the ergopeptine assembly system in the ergot fungus *Claviceps purpurea*. *Chem. Biol.* 10:1281-1292.
- Coyle, C. M., Cheng, J. Z., O'Connor, S. E., and Panaccione, D. G. 2010. An old yellow enzyme gene controls the branch point between *Aspergillus fumigatus* and *Claviceps purpurea* ergot alkaloid pathways. *Appl. Environ. Microbiol.* 76:3898-3903.
- Cvak, L., Jegorov, A., Sedmera, P., Cisarova, I., Cejka, J., Kratochvil, B., and Pakhomova, S. 2005. Norleucine, a natural occurrence in a novel ergot alkaloid gamma-ergokryptinine. *Amino Acids* 29:145-150.
- Cvak, L., Jegorov, A., Sedmera, P., Havlicek, V., Ondracek, J., Husak, M., Pakhomova, S., Kratochvil, B., and Granzin, J. 1994. Ergogaline, a new ergot alkaloid, produced by *Claviceps purpurea*: isolation, identification, crystal structure and molecular conformation. *J. Chem. Soc. Perkin 2*:1861-1865.
- Cvak, L., Minár, J., Pakhomova, S., Ondráček, J., Kratochvil, B., Sedmera, P., Havlicek, V., and Jegorov, A. 1996. Ergoladinine, an ergot alkaloid. *Phytochemistry* 42:231-233.
- de Groot, A. N., van Dongen, P. W., Vree, T. B., Hekster, Y. A., and van Roosmalen, J. 1998. Ergot alkaloids. Current status and review of clinical pharmacology and therapeutic use compared with other oxytocics in obstetrics and gynecology. *Drugs* 56:523-535.
- Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.-M., and Gascuel, O. 2008. Phylogeny.fr: Robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* 36:W465-W469.
- di Menna, M. E., Finch, S. C., Popay, A. J., and Smith, B. L. 2012. A review of the *Neotyphodium lolii/Lolium perenne* symbiosis and its associated effects on animal and plant health, with particular emphasis on ryegrass staggers. *N.Z. Vet. J.* 60:315-328.
- Ding, Y., Williams, R. M., and Sherman, D. H. 2008. Molecular analysis of a 4-dimethylallyltryptophan synthase from *Malbranchea aurantiaca*. *J. Biol. Chem.* 283:16068-16076.
- Edgar, R. C. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32:1792-1797.
- Eleuterius, L. N., and Meyers, S. P. 1977. Alkaloids of *Claviceps* from *Spartina*. *Mycologia* 69:838-840.
- Firn, R. D., and Jones, C. G. 2000. The evolution of secondary metabolism—A unifying model. *Mol. Microbiol.* 37:989-994.
- Flieger, M., Sedmera, P., Vokoun, J., Řiřicová, A., and Řeháček, Z. 1982. Separation of four isomers of lysergic acid α -hydroxyethylamide by liquid chromatography and their spectroscopic identification. *J. Chromatogr. A* 236:441-452.
- Florea, S., Andreeva, K., Machado, C., Mirabito, P. M., and Schardl, C. L. 2009. Elimination of marker genes from transformed filamentous fungi by unselected transient transfection with a Cre-expressing plasmid. *Fungal Genet. Biol.* 46:721-730.
- Florea, S., Phillips, T. D., Panaccione, D. G., Farman, M. L., and Schardl, C. L. 2016. Chromosome-end knockoff strategy to reshape alkaloid profiles of a fungal endophyte. *G3 (Bethesda)* 6:2601-2610.
- Gadberry, M. S., Denard, T. M., Spiers, D. E., and Piper, E. L. 2003. Effects of feeding ergovaline on lamb performance in a heat stress environment. *J. Anim. Sci.* 81:1538-1545.
- Gerhards, N., Matuschek, M., Wallwey, C., and Li, S. M. 2015. Genome mining of ascomycetous fungi reveals their genetic potential for ergot alkaloid production. *Arch. Microbiol.* 197:701-713.
- Gerhards, N., Neubauer, L., Tudzynski, P., and Li, S.-M. 2014. Biosynthetic pathways of ergot alkaloids. *Toxins (Basel)* 6:3281-3295.
- Glatt, H., Eich, E., Pertz, H., Becker, C., and Oesch, F. 1987. Mutagenicity experiments on agroclavines, new natural antineoplastic compounds. *Cancer Res.* 47:1811-1814.
- Goetz, K., Coyle, C., Cheng, J., O'Connor, S., and Panaccione, D. 2011. Ergot cluster-encoded catalase is required for synthesis of chanoclavine-I in *Aspergillus fumigatus*. *Curr. Genet.* 57:201-211.
- Gröger, D., and Floss, H. G. 1998. Biochemistry of ergot alkaloids—Achievements and challenges. *Alkaloids Chem. Biol.* 50:171-218.
- Gunter, S. A., and Beck, P. A. 2004. Novel endophyte-infected tall fescue for growing beef cattle. *J. Anim. Sci.* 82:E75-E82.
- Haarmann, T., Lorenz, N., and Tudzynski, P. 2008. Use of a nonhomologous end joining deficient strain ($\Delta ku70$) of the ergot fungus *Claviceps purpurea* for identification of a nonribosomal peptide synthetase gene involved in ergotamine biosynthesis. *Fungal Genet. Biol.* 45:35-44.
- Haarmann, T., Ortel, I., Tudzynski, P., and Keller, U. 2006. Identification of the cytochrome P450 monooxygenase that bridges the clavine and ergoline alkaloid pathways. *ChemBioChem* 7:645-652.
- Havemann, J., Vogel, D., Loll, B., and Keller, U. 2014. Cyclolization of D-lysergic acid alkaloid peptides. *Chem. Biol.* 21:146-155.
- Heinstein, P. F., Lee, S.-L., and Floss, H. G. 1971. Isolation of dimethylallylpyrophosphate: Tryptophan dimethylallyl transferase from the ergot fungus (*Claviceps spec.*). *Biochem. Biophys. Res. Commun.* 44:1244-1251.
- Hopkins, A. A., Young, C. A., Simpson, W. R., Panaccione, D. G., Mittal, S., and Bouton, J. H. 2010. Agronomic performance and lamb safety of tall fescue novel endophyte combinations in the south central USA. *Crop Sci.* 50:1552-1561.
- Jakubczyk, D., Caputi, L., Hatsch, A., Nielsen, C. A. F., Diefenbacher, M., Klein, J., Molt, A., Schröder, H., Cheng, J. Z., Naesby, M., and O'Connor, S. E. 2015. Discovery and reconstitution of the cycloclavine biosynthetic pathway—Enzymatic formation of a cyclopropyl group. *Angew. Chem. Int. Ed. Engl.* 54:5117-5121.

- Klotz, J. 2015. Activities and effects of ergot alkaloids on livestock physiology and production. *Toxins (Basel)* 7:2801-2821.
- Komarova, E. L., and Tolkachev, O. N. 2001. The chemistry of peptide ergot alkaloids. Part 1. Classification and chemistry of ergot peptides. *Pharm. Chem. J.* 35:504-513.
- Kover, P. X., and Clay, K. 1998. Trade-off between virulence and vertical transmission and the maintenance of a virulent plant pathogen. *Am. Nat.* 152:165-175.
- Leuchtmann, A., Bacon, C. W., Schardl, C. L., White, J. F., and Tadych, M. 2014. Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. *Mycologia* 106:202-215.
- Li, T., Blande, J. D., Gundel, P. E., Helander, M., and Saikkonen, K. 2014. *Epichloë* endophytes alter inducible indirect defences in host grasses. *PLoS ONE* 9:e101331.
- Lorenz, N., Olšovská, J., Šulc, M., and Tudzynski, P. 2010. Alkaloid cluster gene *ccsA* of the ergot fungus *Claviceps purpurea* encodes chanoclavine I synthase, a flavin adenine dinucleotide-containing oxidoreductase mediating the transformation of *N*-methyl-dimethylallyltryptophan to chanoclavine I. *Appl. Environ. Microbiol.* 76:1822-1830.
- Lorenz, N., Wilson, E. V., Machado, C., Schardl, C. L., and Tudzynski, P. 2007. Comparison of ergot alkaloid biosynthesis gene clusters in *Claviceps* species indicates loss of late pathway steps in evolution of *C. fusiformis*. *Appl. Environ. Microbiol.* 73:7185-7191.
- Lyons, P. C., Plattner, R. D., and Bacon, C. W. 1986. Occurrence of peptide and clavine ergot alkaloids in tall fescue grass. *Science* 232:487-489.
- Maier, W., Schumann, B., and Gröger, D. 1988. Microsomal oxygenases involved in ergoline alkaloid biosynthesis of various *Claviceps* strains. *J. Basic Microbiol.* 28:83-93.
- Malinowski, D. P., and Belesky, D. P. 2000. Adaptations of endophyte-infected cool-season grasses to environmental stresses: Mechanisms of drought and mineral stress tolerance. *Crop Sci.* 40:923-940.
- Malinowski, D. P., Belesky, D. P., Hill, N. S., Baligar, V. C., and Fedders, J. M. 1998. Influence of phosphorus on the growth and ergot alkaloid content of *Neotyphodium coenophialum*-infected tall fescue (*Festuca arundinacea* Schreb.). *Plant Soil* 198:53-61.
- Marcet-Houben, M., and Gabaldón, T. 2016. Horizontal acquisition of toxic alkaloid synthesis in a clade of plant associated fungi. *Fungal Genet. Biol.* 86:71-80.
- Marlatt, M. L., West, C. P., McConnell, M. E., Sleper, D. A., Buck, G. W., Correll, J. C., and Saidi, S. 1997. Investigations on xeriphytic *Festuca* spp. from Morocco and their associated endophytes. Pages 73-75 in: *Neotyphodium/Grass Interactions*. C. W. Bacon, and N. S. Hill, eds. Plenum Press, New York and London.
- Matuschek, M., Wallwey, C., Wollinsky, B., Xie, X., and Li, S.-M. 2012. In vitro conversion of chanoclavine-I aldehyde to the stereoisomers festuclavine and pyroclavine controlled by the second reduction step. *RSC Advances* 2:3662-3669.
- McKay, A. C., and Ophel, K. M. 1993. Toxicogenic *Clavibacter/Anguina* associations infecting grass seedheads. *Annu. Rev. Phytopathol.* 31:151-167.
- Metzger, U., Schall, C., Zocher, G., Unsöld, I., Stec, E., Li, S.-M., Heide, L., and Stehle, T. 2009. The structure of dimethylallyl tryptophan synthase reveals a common architecture of aromatic prenyltransferases in fungi and bacteria. *Proc. Natl. Acad. Sci. USA* 106:14309-14314.
- Miedaner, T., and Geiger, H. H. 2015. Biology, genetics, and management of ergot (*Claviceps* spp.) in rye, sorghum, and pearl millet. *Toxins (Basel)* 7: 659-678.
- Mulinti, P., Florea, S., Schardl, C. L., and Panaccione, D. G. 2016. Modulation of ergot alkaloids in a grass-endophyte symbiosis by alteration of mRNA concentrations of an ergot alkaloid synthesis gene. *J. Agric. Food Chem.* 64: 4982-4989.
- Nagabhyru, P., Dinkins, R. D., Wood, C. L., Bacon, C. W., and Schardl, C. L. 2013. Tall fescue endophyte effects on tolerance to water-deficit stress. *BMC Plant Biol.* 13:127.
- Negård, M., Uhlig, S., Kausarud, H., Andersen, T., Høiland, K., and Vrålstad, T. 2015. Links between genetic groups, indole alkaloid profiles and ecology within the grass-parasitic *Claviceps purpurea* species complex. *Toxins (Basel)* 7:1431-1456.
- Ortel, I., and Keller, U. 2009. Combinatorial assembly of simple and complex d-lysergic acid alkaloid peptide classes in the ergot fungus *Claviceps purpurea*. *J. Biol. Chem.* 284:6650-6660.
- Otsuka, H., Quigley, F. R., Gröger, D., Anderson, J. A., and Floss, H. G. 1980. In vivo and in vitro evidence for *N*-methylation as the second pathway-specific step in ergoline biosynthesis. *Planta Med.* 40:109-119.
- Panaccione, D. G. 2005. Origins and significance of ergot alkaloid diversity in fungi. *FEMS Microbiol. Lett.* 251:9-17.
- Panaccione, D. G., Cipoletti, J. R., Sedlock, A. B., Blemings, K. P., Schardl, C. L., Machado, C., and Seidel, G. E. 2006b. Effects of ergot alkaloids on food preference and satiety in rabbits, as assessed with gene-knockout endophytes in perennial ryegrass (*Lolium perenne*). *J. Agric. Food Chem.* 54:4582-4587.
- Panaccione, D. G., Johnson, R. D., Wang, J. H., Young, C. A., Damrongkool, P., Scott, B., and Schardl, C. L. 2001. Elimination of ergovaline from a grass-*Neotyphodium* endophyte symbiosis by genetic modification of the endophyte. *Proc. Natl. Acad. Sci. USA* 98:12820-12825.
- Panaccione, D. G., Kotcon, J. B., Schardl, C. L., Johnson, R. D., and Morton, J. B. 2006a. Ergot alkaloids are not essential for endophytic fungus-associated population suppression of the lesion nematode, *Pratylenchus scribneri*, on perennial ryegrass. *Nematology* 8:583-590.
- Panaccione, D. G., Tapper, B. A., Lane, G. A., Davies, E., and Fraser, K. 2003. Biochemical outcome of blocking the ergot alkaloid pathway of a grass endophyte. *J. Agric. Food Chem.* 51:6429-6437.
- Pažoutová, S., Olšovská, J., Linka, M., Kolínská, R., and Flieger, M. 2000. Chemoraces and habitat specialization of *Claviceps purpurea* populations. *Appl. Environ. Microbiol.* 66:5419-5425.
- Perellino, N. C., Malyszko, J., Ballabio, M., Gioia, B., and Minghetti, A. 1993. Identification of ergobine, a new natural peptide ergot alkaloid. *J. Nat. Prod.* 56:489-493.
- Petroski, R., Powell, R. G., and Clay, K. 1992. Alkaloids of *Stipa robusta* (sleepygrass) infected with an *Acremonium* endophyte. *Nat. Toxins* 1:84-88.
- Pomilio, A. B., Rofi, R. D., Gambino, M. P., Mazzini, C. A., and DeBenedetti de Langenheim, R. T. 1989. The lethal principle of *Poa huachuco* (coirón blanco): A plant indigenous to Argentina. *Toxicol.* 27:1251-1262.
- Plowman, T. C., Leuchtmann, A., Blaney, C., and Clay, K. 1990. Significance of the fungus *Balsania cyperi* infecting medicinal species of *Cyperus* (Cyperaceae) from Amazonia. *Econ. Bot.* 44:452-462.
- Poinar, G. J., Alderman, S., and Wunderlich, J. 2015. One hundred million year old ergot: Psychotropic compounds in the Cretaceous? *Palaeodiversity* 8:13-19.
- Potter, D. A., Stokes, J. T., Redmond, C. T., Schardl, C. L., and Panaccione, D. G. 2008. Contribution of ergot alkaloids to suppression of a grass-feeding caterpillar assessed with gene-knockout endophytes in perennial ryegrass. *Entomol. Exp. Appl.* 126:138-147.
- Powell, R. G., Plattner, R. D., Yates, S. G., Clay, K., and Leuchtmann, A. 1990. Ergobalansine, a new ergot-type peptide alkaloid isolated from *Cenchrus echinatus* (sandbur grass) infected with *Balsania oblecta*, and produced in liquid cultures of *B. oblecta* and *Balsania cyperi*. *J. Nat. Prod.* 53:1272-1279.
- Riley, I. T., Gregory, A. R., Aleen, J. G., and Edgar, J. A. 2003. Poisoning of livestock in Oregon in the 1940s to 1960s attributed to corynetoxins produced by *Rathayibacter* in nematode galls in chewing fescue (*Festuca nigrescens*). *Vet. Hum. Toxicol.* 45:160-162.
- Robinson, S. L., and Panaccione, D. G. 2014. Heterologous expression of lysergic acid and novel ergot alkaloids in *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* 80:6465-6472.
- Robinson, S. L., and Panaccione, D. G. 2015. Diversification of ergot alkaloids in natural and modified fungi. *Toxins (Basel)* 7:201-218.
- Ryan, K. L., Akhmedov, N. G., and Panaccione, D. G. 2015. Identification and structural elucidation of ergotryptamine, a new ergot alkaloid produced by genetically modified *Aspergillus nidulans* and natural isolates of *Epichloë* species. *J. Agric. Food Chem.* 63:61-67.
- Saikkonen, K., Young, C. A., Helander, M., and Schardl, C. L. 2016. Endophytic *Epichloë* species and their grass hosts: From evolution to applications. *Plant Mol. Biol.* 90:665-675.
- Schardl, C. L., Chen, L., and Young, C. A. 2014a. Fungal endophytes of grasses and morning glories, and their bioprotective alkaloids. Pages 125-145 in: *Natural Products: Discourse, Diversity, and Design*. A. Osbourn, R. J. Goss, and G. T. Carter, eds. John Wiley & Sons, Inc., Hoboken, NJ.
- Schardl, C. L., Florea, S., Pan, J., Nagabhyru, P., Bec, S., and Calie, P. J. 2013a. The epichloae: Alkaloid diversity and roles in symbiosis with grasses. *Curr. Opin. Plant Biol.* 16:480-488.
- Schardl, C. L., Leuchtmann, A., and Spiering, M. J. 2004. Symbioses of grasses with seedborne fungal endophytes. *Annu. Rev. Plant Biol.* 55: 315-340.
- Schardl, C. L., Panaccione, D. G., and Tudzynski, P. 2006. Ergot alkaloids—biology and molecular biology. *Alkaloids Chem. Biol.* 63:45-86.
- Schardl, C. L., Young, C. A., Faulkner, J. R., Florea, S., and Pan, J. 2012. Chemotypic diversity of epichloae, fungal symbionts of grasses. *Fungal Ecol.* 5:331-344.
- Schardl, C. L., Young, C. A., Hesse, U., Amyotte, S. G., Andreeva, K., Calie, P. J., Fleetwood, D. J., Haws, D. C., Moore, N., Oeser, B., Panaccione, D. G., Schweri, K. K., Voisey, C. R., Farman, M. L., Jaromczyk, J. W., Roe, B. A., O'Sullivan, D. M., Scott, B., Tudzynski, P., An, Z., Arnaoudova, E. G., Bullock, C. T., Charlton, N. D., Chen, L., Cox, M., Dinkins, R. D., Florea, S., Glenn, A. E., Gordon, A., Güldener, U., Harris, D. R., Hollin, W., Jaromczyk, J., Johnson, R. D., Khan, A. K., Leistner, E., Leuchtmann, A., Li, C., Liu, J., Liu, J., Liu, M., Mace, W., Machado, C., Nagabhyru, P., Pan, J., Schmid, J., Sugawara, K., Steiner, U., Takach, J., Tanaka, E., Webb, J. S., Wilson, E. V., Wiseman, J. L., Yoshida, R., and Zeng, Z. 2013c. Plant-symbiotic

- fungi as chemical engineers: Multi-genome analysis of the Clavicipitaceae reveals dynamics of alkaloid loci. *PLoS Genet.* 9:e1003323.
- Schardl, C. L., Young, C. A., Moore, N., Krom, N., Dupont, P.-Y., Pan, J., Florea, S., Webb, J. S., Jaromczyk, J., Jaromczyk, J. W., Cox, M. P., and Farman, M. L. 2014b. Genomes of plant-associated Clavicipitaceae. *Adv. Bot. Res.* 70:291-327.
- Schardl, C. L., Young, C. A., Pan, J., Florea, S., Takach, J. E., Panaccione, D. G., Farman, M. L., Webb, J. S., Jaromczyk, J., Charlton, N. D., Nagabhyru, P., Chen, L., Shi, C., and Leuchtmann, A. 2013b. Currencies of mutualisms: Sources of alkaloid genes in vertically transmitted epichloae. *Toxins (Basel)* 5:1064-1088.
- Schumann, G. L. 2000. Ergot. Updated 2005. The Plant Health Instructor. Published online. doi:10.1094/PHI-I-2000-1016-01
- Ščigelová, M., Macek, T., Minghetti, A., Macková, M., Sedmera, P., Přikrylová, V., and Křen, V. 1995. Biotransformation of ergot alkaloids by plant cell cultures with high peroxidase activity. *Biotechnol. Lett.* 17:1213-1218.
- Shymanovich, T., Saari, S., Lovin, M. E., Jarmusch, A. K., Jarmusch, S. A., Musso, A. M., Charlton, N. D., Young, C. A., Cech, N. B., and Faeth, S. H. 2015. Alkaloid variation among epichloid endophytes of sleepygrass (*Achnatherum robustum*) and consequences for resistance to insect herbivores. *J. Chem. Ecol.* 41:93-104.
- Spatafora, J. W., and Bushley, K. E. 2015. Phylogenomics and evolution of secondary metabolism in plant-associated fungi. *Curr. Opin. Plant Biol.* 26:37-44.
- Spiers, D. E., Zhang, Q., Eichen, P. A., Rottinghaus, G. E., Garner, G. B., and Eilersieck, M. R. 1995. Temperature-dependent responses of rats to ergovaline derived from endophyte-infected tall fescue. *J. Anim. Sci.* 73:1954-1961.
- Takach, J. E., Mittal, S., Swoboda, G. A., Bright, S. K., Trammell, M. A., Hopkins, A. A., and Young, C. A. 2012. Genotypic and chemotypic diversity of *Neotyphodium* endophytes in tall fescue from Greece. *Appl. Environ. Microbiol.* 78:5501-5510.
- Thompson, F. N., and Stuedemann, J. A. 1993. Pathophysiology of fescue toxicosis. *Agric. Ecosyst. Environ.* 44:263-281.
- Timper, P., Gates, R. N., and Bouton, J. H. 2005. Response of *Pratylenchus* spp. in tall fescue infected with different strains of the fungal endophyte *Neotyphodium coenophialum*. *Nematology* 7:105-110.
- Tsai, H.-F., Wang, H., Gebler, J. C., Poulter, C. D., and Schardl, C. L. 1995. The *Claviceps purpurea* gene encoding dimethylallyltryptophan synthase, the committed step for ergot alkaloid biosynthesis. *Biochem. Biophys. Res. Commun.* 216:119-125.
- van Dongen, P. W., and de Groot, A. N. 1995. History of ergot alkaloids from ergotism to ergometrine. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 60:109-116.
- Vazquez, M. J., Roa, A. M., Reyes, F., Vega, A., Rivera-Sagredo, A., Thomas, D. R., Diez, E., and Hueso-Rodriguez, J. A. 2003. A novel ergot alkaloid as a 5-HT(1A) inhibitor produced by *Dicyma* sp. *J. Med. Chem.* 46:5117-5120.
- Wallwey, C., Heddergott, C., Xie, X., Brakhage, A. A., and Li, S.-M. 2012. Genome mining reveals the presence of a conserved biosynthetic gene cluster for the biosynthesis of ergot alkaloid precursors in the fungal family Arthrodermataceae. *Microbiology* 158:1634-1644.
- Wallwey, C., and Li, S. M. 2011. Ergot alkaloids: Structure diversity, biosynthetic gene clusters and functional proof of biosynthetic genes. *Nat. Prod. Rep.* 28:496-510.
- Wallwey, C., Matuschek, M., and Li, S.-M. 2010. Ergot alkaloid biosynthesis in *Aspergillus fumigatus*: Conversion of chanoclavine-I to chanoclavine-I aldehyde catalyzed by a short-chain alcohol dehydrogenase FgaDH. *Arch. Microbiol.* 192:127-134.
- Walton, J. D. 2000. Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: An hypothesis. *Fungal Genet. Biol.* 30:167-171.
- Wang, J., Machado, C., Panaccione, D. G., Tsai, H.-F., and Schardl, C. L. 2004. The determinant step in ergot alkaloid biosynthesis by an endophyte of perennial ryegrass. *Fungal Genet. Biol.* 41:189-198.
- Young, C. A., Schardl, C. L., Panaccione, D. G., Florea, S., Takach, J. E., Charlton, N. D., Moore, N., Webb, J. S., and Jaromczyk, J. 2015. Genetics, genomics and evolution of ergot alkaloid diversity. *Toxins (Basel)* 7:1273-1302.