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Heterologous Expression of Lysergic Acid and Novel Ergot Alkaloids in *Aspergillus fumigatus*

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Different lineages of fungi produce distinct classes of ergot alkaloids. Lysergic acid-derived ergot alkaloids produced by fungi in the Clavicipitaceae are particularly important in agriculture and medicine. The pathway to lysergic acid is partly elucidated, but the gene encoding the enzyme that oxidizes the intermediate agroclavine is unknown. We investigated two candidate agroclavine oxidase genes from the fungus *Epichloë festucae* var. *lolii* × *Epichloë typhina* isolate Lp1 (henceforth referred to as *Epichloë* sp. Lp1), which produces lysergic acid-derived ergot alkaloids. Candidate genes *easH* and *cloA* were expressed in a mutant strain of the mold *Aspergillus fumigatus*, which typically produces a subclass of ergot alkaloids not derived from agroclavine or lysergic acid. Candidate genes were coexpressed with the *Epichloë* sp. Lp1 allele of *easA*, which encodes an enzyme that catalyzed the synthesis of agroclavine from an *A. fumigatus* intermediate; the agroclavine then served as the substrate for the candidate agroclavine oxidases. Strains expressing *easA* and *cloA* from *Epichloë* sp. Lp1 produced lysergic acid from agroclavine, a process requiring a cumulative six-electron oxidation and a double-bond isomerization. Strains that accumulated excess agroclavine (as a result of *Epichloë* sp. Lp1 *easA* expression in the absence of *cloA*) metabolized it into two novel ergot alkaloids for which provisional structures were proposed on the basis of mass spectra and precursor feeding studies. Our data indicate that CloA catalyzes multiple reactions to produce lysergic acid from agroclavine and that combining genes from different ergot alkaloid pathways provides an effective strategy to engineer important pathway molecules and novel ergot alkaloids.

Ergot alkaloids are agriculturally and pharmaceutically relevant secondary metabolites synthesized by several species of fungi. Historically, ergot alkaloids caused periodic mass poisonings due to infection of grain crops by the ergot fungus *Claviceps purpurea* (1). Agriculturally, ergot alkaloids in forage grasses colonized by endophytic *Epichlë* spp. (including many fungi recently realigned from the genus *Neotyphodium* [2]) reduce weight gain and fitness in grazing animals (3, 4). Clinically, the structural similarities of ergot alkaloids to neurotransmitters allow ergot alkaloids to treat cognitive and neurological maladies, including dementia, migraines, and Parkinson's disease, in addition to endocrine disorders such as type 2 diabetes and hyperprolactinemia (5–8). Indeed, the neurotransmitter-mimicking activities of ergot alkaloids are most infamously evident in the psychoactive drug lysergic acid diethylamide (LSD), a semisynthetic ergot alkaloid derivative.

Representatives of two major families of fungi—the Clavicipitaceae and the Trichocomaceae—produce ergot alkaloids. All ergot alkaloid-producing fungi share early pathway steps before diverging to produce lineage-specific classes of ergot alkaloids (Fig. 1). Members of the Clavicipitaceae, including *Claviceps purpurea* and the endophytic *Epichloë* species such as *E. festucae* var. *lolii* × *E. typhina* isolate Lp1 (2, 9) (henceforth called *Epichloë* sp. Lp1), synthesize lysergic acid-based alkaloids in which the D ring of the ergoline nucleus is unsaturated between carbons 9 and 10 and carbon 17 is highly oxidized (Fig. 1) (4, 10, 11). Ergot alkaloid-producing fungi in the Trichocomaceae, such as the opportunistic human pathogen *Aspergillus fumigatus*, produce clavine-based derivatives in which the D ring is saturated and carbon 17 remains reduced as a methyl group (12, 13).

The branch point of the pathway occurs during D ring closure. In *A. fumigatus*, the 8,9 double bond in chanoclavine aldehyde is reduced by the enzyme EasA reductase, allowing the aldehyde group free rotation to interact with the secondary amine to promote ring closure via Schiff base formation (14–16). The resulting iminium ion is subsequently reduced by EasG to form festuclavine (16, 17), which may be modified at carbons 9 and/or 2 to form various fumigaclavine derivatives (Fig. 1). Most ergot alkaloid-producing fungi in the Clavicipitaceae, however, synthesize the 8,9 unsaturated clavine agroclavine from chanoclavine aldehyde via the activity of an alternate version of EasA that acts as an isomerase rather than a reductase (15, 17). In *C. purpurea* and *Epichloë* spp., agroclavine is oxidized to form elymoclavine, and elymoclavine is further oxidized and isomerized to form lysergic acid (Fig. 1). Lysergic acid is then incorporated into ergopeptines and/or lysergic acid amides. Lysergic acid derivatives are the ergot alkaloids used for pharmaceutical development, but these compounds are produced exclusively in clavicipitaceous fungi and not in model organisms that would facilitate their modification and development.

The genetics of many steps in the ergot alkaloid pathway have been characterized; however, the identity of the gene encoding the oxidase that converts agroclavine to elymoclavine has remained elusive. All known genes involved in ergot alkaloid synthesis (*eas* genes) in both *A. fumigatus* and the Clavicipitaceae have been found in clusters (Fig. 2) (11, 18–24). The roles of many genes in *eas* clusters have been determined by gene knockout or by expression of coding sequences in *Escherichia coli*. Among the genes in

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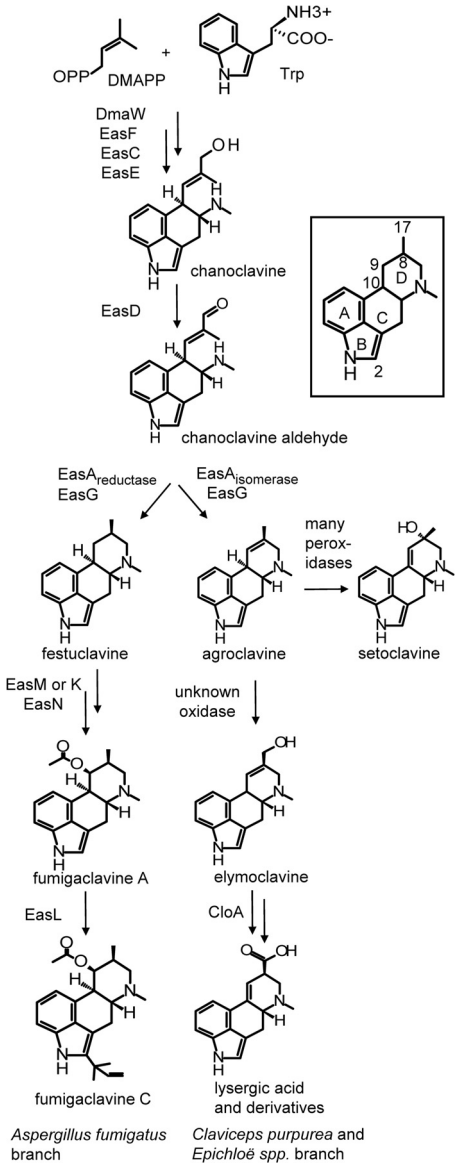

FIG 1 Intermediates and products of the ergot alkaloid pathway (as composited from branches found in different fungi). The role of different alleles of *easA* (isomerase versus reductase encoding types) in controlling the branch point is indicated. Alkaloids with a 9,10 double bond (e.g., setoclavine and lysergic acid and its derivatives) often occur as diastereoisomers at position 8. Roles for enzymes discussed in the text or with genes illustrated in Fig. 2 are indicated near arrows. Double arrows indicate one or more omitted intermediates. The inset shows ring and position labeling referred to in the text. DMAPP, dimethylallylpyrophosphate.

eas clusters of lysergic acid-producing fungi, two genes stand out as candidates to encode the enzyme that oxidizes agroclavine. The gene labeled *easH* encodes a product with high similarity to dioxygenases (10, 19, 25); at the time this work was conducted, its role in the pathway had not been tested, but very recently EasH has been demonstrated to oxidize lysergyl-peptide lactams to facilitate their cyclolization to ergopeptines (25). This gene is present in both *A. fumigatus* and *Epichloë* spp.; however, the copy found in *A. fumigatus* (which lacks agroclavine and lysergic acid derivatives) is a pseudogene (10). The gene named *cloA*, for clavine oxidase (26), encodes a P450 monooxygenase that is a second candidate. Haarmann et al. (26) showed that CloA was required for oxidation of carbon 17 of elymoclavine during synthesis of lysergic acid and speculated that CloA also oxidized the same carbon in agroclavine. Only fungi that produce lysergic acid-derived alkaloids contain *cloA* in their *eas* clusters (18–24).

To test each candidate gene, a heterologous expression system was designed using an *A. fumigatus easA* knockout (*easA* KO) (15) as the host strain, which allowed for precise pathway control based on insertion of an agroclavine-specific allele of *easA*. Constructs used for transformation contained three elements: *easA* from *Epichloë* sp. Lp1, a bidirectional *easA-easG* promoter from *A. fumigatus*, and the candidate gene (either *easH* or *cloA*) amplified from *Epichloë* sp. Lp1 (Fig. 2). Coexpression of the *Epichloë* sp. Lp1 isomerase allele of *easA* in the *easA* reductase knockout background of *A. fumigatus* allowed accumulation of agroclavine (15, 17), which served as the substrate for the enzyme expressed from the candidate gene in the construct. This combinatorial approach allowed us to emulate the lysergic acid pathway in *A. fumigatus* and identify the gene encoding the agroclavine-oxidizing enzyme. Moreover, production of agroclavine in *A. fumigatus* in the absence of its oxidizing enzyme allowed accumulation of novel ergot alkaloids as a result of the activity of native *A. fumigatus* enzymes on agroclavine.

MATERIALS AND METHODS

Preparation of transformation constructs. Each candidate oxidase gene (*easH* or *cloA*) was incorporated into a three-component construct that contained a bidirectional promoter from *A. fumigatus* (originating from the divergently transcribed genes *easA* and *easG*) centered between the candidate oxidase gene from *Epichloë* sp. Lp1 and the allele of *easA* from *Epichloë* sp. Lp1 (Fig. 2). To generate the *easA*-bidirectional promoter-*easH* construct, the following PCR amplifications were performed (Table 1). The *easA* portion was amplified from *Epichloë* sp. Lp1 DNA with primer combination 1 and Phire Hot Start II polymerase (Thermo Fisher, Waltham, MA). The initial denaturation was at 98°C for 30 s, followed by 35 cycles of 98°C (15 s), 60°C (15 s), and 72°C (30 s) and a final extension at 72°C for 60 s. The bidirectional *easA-easG* promoter of *A. fumigatus* was amplified from template DNA of *A. fumigatus* wild-type strain Af 293 by application of the PCR protocol described above for *easA* but with primer combination 2. Coding sequences and the 3′ untranslated region of *easH* were amplified from *Epichloë* sp. Lp1 DNA with the same PCR protocol as *easA*; however, the *easH* reaction was primed with primer combination 3. After each PCR, the products were cleaned with QIAquick columns (Qiagen, Gaithersburg, MD). To combine *easA*, the bidirectional promoter, and *easH* into a single construct, approximately equimolar amounts of each of the three PCR products were combined in a fusion PCR with Phire polymerase and primer combination 4. The PCR protocol had an initial denaturation at 98°C for 30 s, followed by 35 cycles of 98°C (15 s), 60°C (15 s), and 72°C (45 s) and a final elongation at 72°C for 60 s. The fusion product was purified by agarose gel electrophoresis and Qiaquick column chromatography prior to fungal transformation. The integrity of the construct was confirmed by DNA sequence analysis.

Generation of the construct containing *Epichloë* sp. Lp1 *easA*, the bidirectional promoter from *A. fumigatus*, and *Epichloë* sp. Lp1 *cloA* was similar to that described above for the *easA-easH* construct. However, the primers for each fragment were the following: combination 1 for *easA*, combination 5 for the promoter, and combination 6 for *cloA* (Table 1). Moreover, a different PCR protocol was used for the fusion reaction. This Phire reaction included primer combination 7 and had an initial denaturation temperature of 98°C for 30 s, followed by 35 cycles of 98°C (15 s), 62°C (15 s), and 72°C (65 s) and ending with an elongation period of 72°C for 60 s. The integrity of the construct was verified by DNA sequencing.

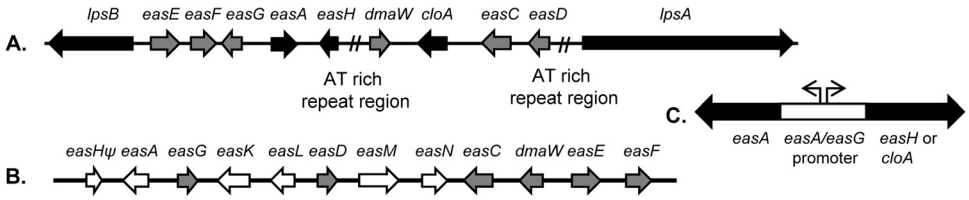

FIG 2 Ergot alkaloid synthesis (*eas*) clusters from *Epichloë* sp. Lp1 (A) and *Aspergillus fumigatus* (B) and design of transformation construct (C). (A) *Epichloë* sp. Lp1 *eas* cluster redrawn from the work of Schardl et al. (24); two AT-rich repeat regions (15 to 25 kb each) were compressed in the diagram to facilitate the presentation. (B) *Aspergillus fumigatus eas* cluster redrawn from the works of Unsöld and Li (22) and Coyle and Panaccione (18). Ψ, pseudogene. Genes unique to the *Epichloë* sp. Lp1 cluster are shown in black, and those unique to the *A. fumigatus* cluster are indicated in white. Genes common to both clusters are shown in gray. Although both clusters contain an allele of *easA*, the products of those alleles differ functionally, and so they differ in shading in their respective clusters. (C) General design of constructs generated by fusion PCR. Candidate genes were *cloA* or *easH* from *Epichloë* sp. Lp1. Black and white fragments correspond to *Epichloë* sp. Lp1 or *A. fumigatus* origin, as described above.

Fungal transformation. Candidate oxidase constructs were cotransformed into the *A. fumigatus easA* KO strain (15), along with the selectable marker pAMD1, which contains the acetamidase gene of *Aspergillus nidulans* (27). Transformants capable of utilizing acetamide as a source of nitrogen were selected on acetamide medium (28). The spheroplast-polyethylene glycol (PEG)-based transformation protocol was based on previously described methods (15, 18).

mRNA analyses. Cultures were grown in 50 ml of malt extract broth (Difco, Detroit, MI) in a 250-ml flask for 1 day with shaking at 80 rpm at 37°C to form a mat of hyphae on the surface of the broth. The mat was transferred to an empty petri dish and incubated at 37°C for an additional day to promote conidiation. RNA was extracted from approximately 100 mg of a conidiating colony with the Plant RNeasy kit (Qiagen), treated with DNase I (Qiagen), and reverse transcribed with Superscript II (Invitrogen, Carlsbad, CA). Template cDNA from each class of transformant was diluted 1:1,000 prior to amplification, and the presence of transcripts from individual genes was tested by PCR with primers (Table 1) specific for *easA* (combination 8), *easH* (combination 9), and *cloA* (combination 10). PCR was conducted with Phire polymerase in the following program: 98°C for 30 s, followed by 35 cycles of 98°C (10 s), 60°C (10 s), and 72°C (15 s), finishing with an elongation at 72°C for 60 s. RNA extraction and subsequent cDNA synthesis for all strains were performed concurrently to ensure consistency. The absence of genomic DNA in the *easA-cloA* cDNA preparation was confirmed by priming amplification with oligonucleotides that flank an intron, such that a second, larger product would be amplified from any contaminating genomic DNA. Of the three genes tested, only *cloA* contains introns.

Alkaloid analyses. For quantitative analyses, six replicate colonies of one transformant of each type were grown on malt extract agar (15 g of malt extract broth medium plus 15 g of agar per liter) for 11 days. Samples of an approximately 50-mm^2 surface area were collected with the broad end of a 1,000-μl pipette tip. Unless otherwise indicated, alkaloids were extracted with 98% methanol plus 2% acetic acid at 55°C for 30 min. Alternate extractions were conducted with 100% methanol or 10% aqueous ammonium acetate. Conidia in each extract were counted to provide an estimate of fungal biomass. Extracts clarified by centrifugation were then analyzed by reverse-phase high-performance liquid chromatography (HPLC) with fluorescence detection (12). Briefly, samples were separated on a 150-mm by 4.6-mm C_{18} column (Phenomenex Prodigy ODS3, 5-μm particle size; Torrance, CA) with a multilinear binary gradient of 5% acetonitrile plus 50 mM ammonium acetate to 75% acetonitrile plus 50 mM ammonium acetate. Lysergic acid standard was prepared by hydrolyzing 1 mg of ergotamine tartrate (Sigma-Aldrich, St. Louis, MO) in 100 μl of 1.2 M NaOH at 75°C for 6 h, followed by neutralization with a 1.2 M solution of HCl, purification on a C_{18} SPE column (Biotage, Charlotte, NC), and verification by liquid chromatography-mass spectrometry (LC/MS).

TABLE 1 PCR primers used in this study and their products

Primer combination	Primer sequences (5′→3′)	Product	Amplicon length(s) (bp)
1	TACTTGGTGGATTAGAAGCAATGTCAACTTCAAATCTTTTCAC + GCCATCATGACACCATTTGA	*easA* with promoter extension[a]	1,841
2	GTGAAAAGATTTGAAGTTGACATTGCTTCTAATCCACCAAGTA + GGCTTGGATTGAACGGTCATGGTGCGGAGTGCCTACTCTA	Promoter with *easA* and *easH* extensions	830
3	TAGAGTAGGCACTCCGCACCATGACCGTTCAATCCAAGCC + CCTAGCTATCCATGCTCAAGC	*easH* with promoter extension	1,135
4	CGTATCACCGAGACAAAGAGG + TTGGCCATCACCTAACTATCTTG	*easA*-promoter-*easH* fusion construct	3,250
5	GTGAAAAGATTTGAAGTTGACATTGCTTCTAATCCACCAAGTA + GGATAACCATGGTAATATCATGGTGCGGAGTGCCTACTCTATAG	Promoter with *easA* and *cloA* extensions	831
6	CTATAGAGTAGGCACTCCGCACCATGATATTACCATGGTTATCC + AACACGCTAAGGGCAACAAG	*cloA* with promoter extension	2,746
7	CGTATCACCGAGACAAAGAGG + GCAACAAGCGATAAGCGTTAG	*easA*-promoter-*cloA* fusion construct	4,905
8	GCGAATGGATTTGATCTCGT + CCAGCGAGAGTTAGCAAGGT	*easA* internal sequences	447
9	CCAACGGTTCTCCCTTACTTC + GCACTATCTTGCCGCTCAGT	*easH* internal sequences	611
10	TTCCCGGCACGAGCTTTGCG + CTTAGAGTGCACCTCAGACGAC	*cloA* internal sequences	296 (cDNA), 362 (gDNA[b])

[a] Extension refers to incorporation of an additional 20 to 23 nucleotides at the 5′ end of a primer to add sequences that will facilitate a later fusion PCR.
[b] gDNA, genomic DNA.

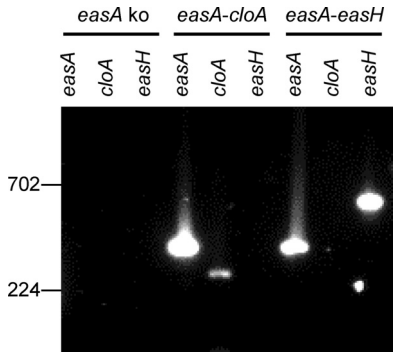

FIG 3 Qualitative reverse transcription-PCR (RT-PCR) demonstrating accumulation of mRNA from the indicated genes in *A. fumigatus easA* KO transformants. For the horizontal strain labels, *easA* ko refers to the nontransformed recipient strain and *easA-cloA* and *easA-easH* refer to transformants. Vertical gene labels refer to the *Epichloë* sp. Lp1 gene for which amplification was attempted in that lane. Primers are listed in Table 1. Each cDNA preparation was diluted 1:1,000 prior to amplification. The scale at the left indicates the relative mobility of relevant fragments from BstEII-digested bacteriophage lambda.

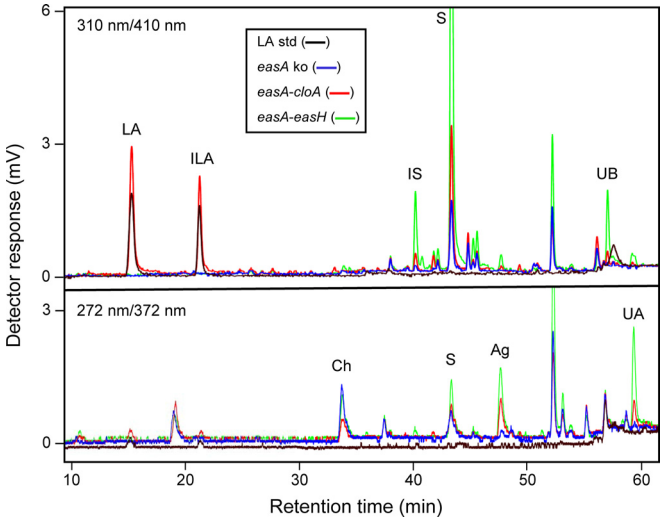

FIG 4 Qualitative analysis of ergot alkaloids from transformed strains of *A. fumigatus*. Samples were analyzed with two fluorescence detectors; excitation and emission wavelengths are indicated. Lysergic acid and other ergot alkaloids with a 9,10 double bond fluoresce more strongly at 310 and 410 nm, whereas other ergot alkaloids fluoresce maximally with settings of 272 and 372 nm (12). Ergot alkaloids with 9,10 double bonds form diastereoisomers at carbon 8. Values for both diastereoisomers were added in quantitative analyses. Strain names and line colors are indicated in the key. Abbreviations: LA, lysergic acid; ILA, isolysergic acid; IS, isosetoclavine; S, setoclavine; UB, unknown B; Ch, chanoclavine; Ag, agroclavine; UA, unknown A. The peak eluting at 53 min is present in nontransformed *A. fumigatus* (15), and its fluorescence ratio indicates that it is not an ergot alkaloid (12).

Chanoclavine was obtained from Alfarma (Prague, Czech Republic), agroclavine was obtained from Fisher (Pittsburgh, PA), and setoclavine was prepared by oxidizing agroclavine as described previously (29, 30).

Ergot alkaloids were quantified by comparing peak areas to standard curves prepared from external standards of ergonovine (for alkaloids fluorescing at 310 nm and 410 nm) and agroclavine (for alkaloids fluorescing at 272 nm and 372 nm) and normalizing to the number of conidia extracted. Quantities of alkaloids among strains were compared by analysis of variance (ANOVA), and when ANOVA indicated a significant effect of fungal strain on alkaloid quantity ($P < 0.05$), means were separated by a Tukey-Kramer test with α set at 0.05. Statistical analyses were performed with JMP (SAS, Cary, NC).

For LC/MS analysis, cultures were grown for 1 week on malt extract agar. Conidiating cultures were washed repeatedly with 4 ml of HPLC-grade methanol. After pelleting of conidia and mycelia by centrifugation, the supernatant was concentrated to 100 μl in a vacuum centrifuge, and 10 μl was analyzed by LC/MS. Samples containing novel ergot alkaloids were analyzed as described previously (31) on a Thermo Fisher LCQ DecaXP LC/MS, whereas lysergic acid samples were analyzed on a Thermo Fisher Q Exactive ultrahigh-performance liquid chromatograph-mass spectrometer (UHPLC/MS) with electrospray ionization in positive mode, a spray voltage of 3.5 kV, and a capillary temperature of 300°C. Samples were chromatographed on a 50- by 2.1-mm Hypersil Gold column with a 1.9-μm particle size (Thermo Fisher) in a linear gradient of 100% solution A (5% acetonitrile plus 0.1% formic acid) to 100% solution B (75% acetonitrile plus 0.1% formic acid) over 10 min at a flow rate of 300 μl/min. Lysergic acid was eluted at approximately 3.3 min. After an initial full scan of *m/z* 50 to 570, ions of *m/z* 269 were fragmented and analyzed.

Precursor feeding study. The ability of strains of *A. fumigatus* to convert agroclavine or setoclavine into novel ergot alkaloids was tested by feeding agroclavine to *A. fumigatus* strains. Agroclavine is readily oxidized to setoclavine by *A. fumigatus* (15, 29) and many other organisms (29, 30). Strains tested included *A. fumigatus* NRRL 164, which lacks a functional copy of the prenyl transferase gene *easL*, and the *A. fumigatus easA* KO strain and *A. fumigatus* Af 293, which have functional copies of *easL* (32). Six replicate cultures of each strain were grown from 60,000 conidia in 200 μl of malt extract broth in a 2-ml microcentrifuge tube. Cultures were supplemented with 37 nmol of agroclavine in 1 μl of methanol or with 1 μl of methanol as a control. An additional control was malt extract broth without conidia but with 1 μl of agroclavine (37 nmol). The cultures were incubated for 1 week at 37°C and then extracted by the addition of 300 μl of methanol along with 10 3-mm-diameter glass beads, followed by bead beating in a Fastprep 120 (Bio101, Carlsbad, CA) at 6 m/s for 30 s. Alkaloids were analyzed by HPLC with fluorescence detection as described above.

RESULTS

The *Aspergillus fumigatus easA* KO strain was successfully transformed with constructs for expressing either *easA-easH* or *easA-cloA* of *Epichloë* sp. Lp1. Evidence of successful transformation and expression of the *Epichloë* sp. Lp1 genes included accumulation of mRNA from both *Epichloë* sp. Lp1 genes introduced with a particular construct (Fig. 3). Further evidence of successful expression of the introduced genes was the altered ergot alkaloid profiles observed by HPLC with fluorescence detection (Fig. 4; Table 2). As described previously (15), the recipient strain, *easA* KO *A. fumigatus*, accumulated primarily chanoclavine and also small quantities of agroclavine (arising via a noncatalyzed isomerization of chanoclavine aldehyde [15, 33]) and larger quantities of its oxidation product setoclavine (Fig. 1). Transformants expressing *Epichloë* sp. Lp1 *easA-easH* accumulated chanoclavine and significantly more agroclavine and setoclavine than did the nontransformed recipient strain ($P < 0.05$), indicating successful expression of the *easA* allele of *Epichloë* sp. Lp1 without further modification of the ergot alkaloid profile by the product of *easH*. The same ergot alkaloid profile was observed in a previous study in which *C. purpurea easA* was expressed in the *A. fumigatus easA* KO strain (15). Strains that expressed the *Epichloë* sp. Lp1 *easA-cloA* construct also accumulated chanoclavine, agroclavine, and setoclavine but at levels comparable to those of the parent strain, *easA* KO *A. fumigatus* (Table 2). In addition, the *easA-cloA*-expressing

TABLE 2 Ergot alkaloid accumulation in cultures of modified strains of *A. fumigatus*

Relevant genotype	Amt accumulated (amol/conidium)[a]					
	Chanoclavine	Agroclavine	Setoclavine[b]	Lysergic acid[b]	Unknown A	Unknown B
easA KO	0.42 ± 0.04	0.055 ± 0.01 B	0.16 ± 0.01 B	ND[c]	ND	ND
easA-cloA	0.58 ± 0.1	0.27 ± 0.04 B	0.60 ± 0.07 B	1.0 ± 0.1	0.16 ± 0.04 B	0.062 ± 0.01 B
easA-easH	0.59 ± 0.07	0.81 ± 0.1 A	2.0 ± 0.2 A	ND	0.38 ± 0.06 A	0.22 ± 0.05 A

[a] Data are means for six cultures of the same strain ± SE; values followed by a different letter within a column differ significantly ($\alpha = 0.05$) in a Tukey-Kramer test.
[b] Values calculated from sums of both diastereoisomers.
[c] ND, not detected; limit of detection = 0.01 amol/conidium.

strains accumulated a pair of polar compounds that were coeluted with lysergic acid and isolysergic acid standards (Fig. 4). The identity of the compounds as lysergic acid and its diastereoisomer was supported by UHPLC/MS analyses in which the *easA-cloA* strains produced parent ion and fragments consistent with those arising from the lysergic acid standard (Fig. 5). These data indicate that CloA catalyzes a cumulative six-electron oxidation of agroclavine to lysergic acid and that CloA or EasA isomerizes the 8,9 double bond in the D ring to the 9,10 position. The amount of lysergic acid extracted from *easA-cloA* strain cultures varied depending on the solvent used. Both 98% methanol plus 2% acetic acid and 10% (wt/vol) aqueous ammonium carbonate extracted significantly more (approximately 2.5-fold) lysergic acid than did unsupplemented methanol ($P < 0.05$).

In addition to known ergot alkaloids described above, strains transformed with constructs containing either *easA-easH* or *easA-cloA* fragments accumulated two novel alkaloids referred to as unknown A and unknown B (Fig. 4; Table 2). The *easA-easH*-expressing strain, which accumulated significantly more agroclavine and setoclavine than did the *easA-cloA*-expressing strain, also accumulated significantly greater quantities of unknowns A and B ($P < 0.05$). Unknown A fluoresced more intensely at the 272- and 372-nm wavelength settings than at the 310- and 410-nm wavelength settings (Fig. 4), which is typical of ergot alkaloids lacking a double bond between positions 9 and 10 of the ergoline nucleus (12). In contrast, unknown B fluoresced more intensely at the 310- and 410-nm wavelengths than at the 272- and 372-nm wavelengths, indicating the presence of a 9,10 double bond (12). LC/MS analyses revealed that unknowns A and B had molecular ions with masses of 307.3 and 323.2, respectively (Fig. 6). The molecular ion of unknown A corresponds to the mass of [agroclavine + H]$^+$ with an additional prenyl group, whereas the molecular ion of unknown B corresponds to the mass of [setoclavine + H]$^+$ with an additional prenyl group.

To test the hypothesis that unknowns A and B correspond, respectively, to prenylated versions of agroclavine and setoclavine, agroclavine was fed to three isolates of *A. fumigatus*: (i) *easA* KO *A. fumigatus*, the transformation recipient, which was derived from *A. fumigatus* isolate FGSC A1141 and contains a functional copy of the ergot alkaloid prenyl transferase gene *easL* (32); (ii) *A. fumigatus* Af 293, a wild-type isolate that also has a functional copy of *easL* (32); and (iii) *A. fumigatus* NRRL 164, an isolate unable to produce the prenylated ergot alkaloid fumigaclavine C due to a mutation resulting in a premature stop codon in *easL* (32). Agroclavine-fed cultures of all isolates contained some nonmetabolized agroclavine and its oxidation product setoclavine. However, only *easA* KO *A. fumigatus* and *A. fumigatus* Af 293, which contain functional copies of the prenyl transferase gene *easL*, accumulated unknowns A and B (Table 3). These data demonstrate a dependency on agroclavine for synthesis of the unknowns and are consistent with a role for the prenyl transferase encoded by *easL* in their biosynthesis.

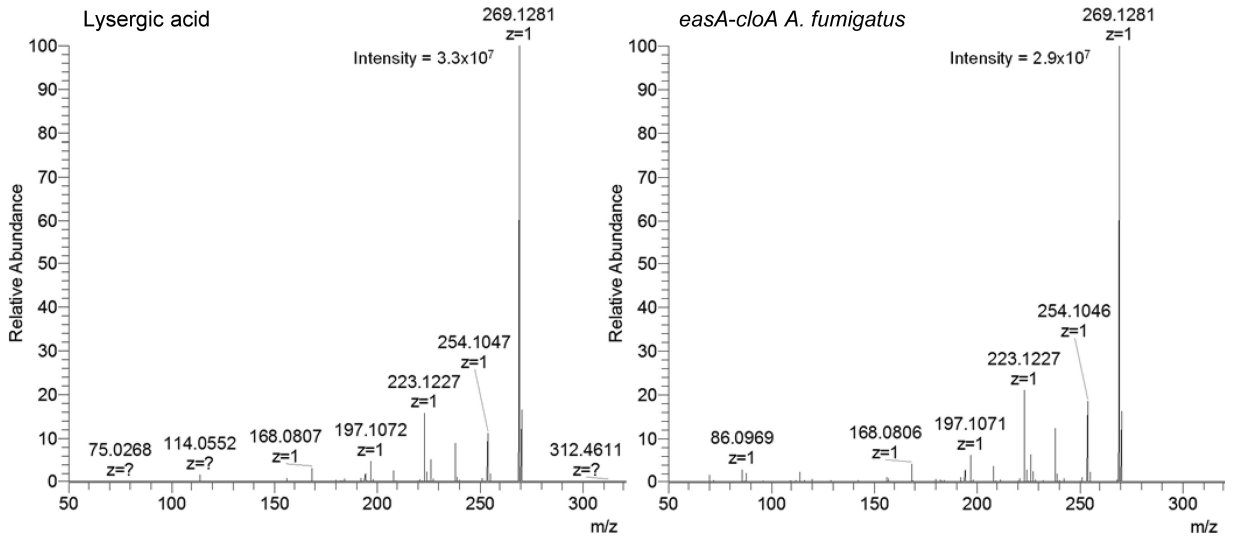

FIG 5 Mass spectra of lysergic acid standard and coeluting analyte from *easA-cloA*-transformed *easA* KO *A. fumigatus*. Spectra were collected with electrospray ionization in positive mode.

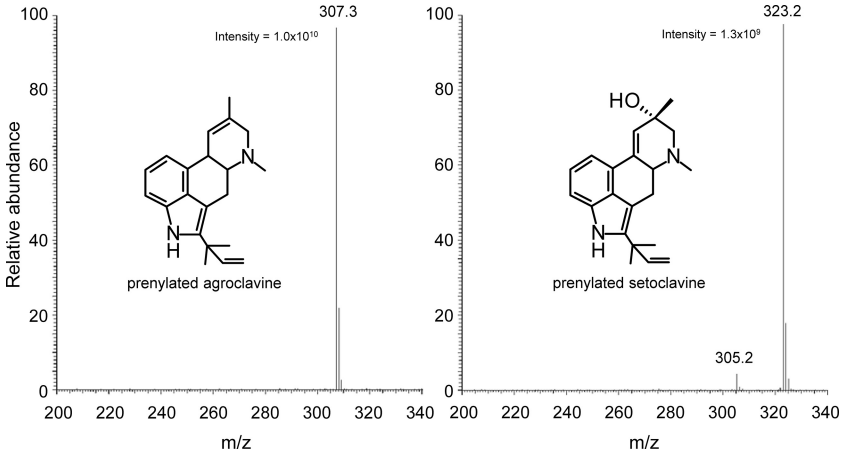

FIG 6 Mass spectra of two unknown alkaloids with hypothesized structures. Spectra were collected from LC/MS analyses with electrospray ionization in positive mode. The position and reverse mode of prenylation are hypothesized based on previously characterized activities of EasL (FgaPT1) (36) and on several closely related alkaloids from *A. fumigatus* (36, 37). Genetic evidence of prenylation is presented in Table 3.

DISCUSSION

By expressing CloA along with the isomerase form of EasA of *Epichloë* sp. Lp1 in an *easA* reductase knockout mutant of *A. fumigatus*, we have reprogrammed *A. fumigatus* to emulate the ergot alkaloid pathway of lysergic acid-producing fungi. Consistent with the data of Coyle et al. (15), both *easA-easH*-expressing and *easA-cloA*-expressing mutants produced agroclavine, as a result of expressing the isomerase form of *Epichloë* sp. Lp1 *easA*. In addition, both types of transformants accumulated setoclavine and isosetoclavine, diastereoisomers formed by the oxidation of agroclavine by endogenous peroxidases in *A. fumigatus* and dozens of other fungi and plants (15, 29, 30). However, comparison of the *easA-cloA*-expressing strain with the *easA-easH*-expressing strain provides evidence of an extended, multistep role for CloA in biosynthesis of lysergic acid. The data indicate that CloA performs multiple catalytic steps: a two-electron oxidation of agroclavine to elymoclavine and then a pair of two-electron oxidations to convert elymoclavine to lysergic acid, presumably via an undetected aldehyde intermediate (34). The role of CloA in catalyzing multiple oxidations to form paspalic acid or lysergic acid was previously hypothesized by Haarmann et al. (26). Our data also suggest that CloA or the coexpressed EasA catalyzes the double-bond isomerization, from position 8,9 to 9,10.

The lack of detectable elymoclavine, or any other intermediates in the oxidation series from agroclavine to lysergic acid, in our positive transformants indicates that CloA may bind agroclavine and execute successive oxidations and an isomerization before releasing lysergic acid. The lack of detectable paspalic acid (which is the 8,9 double-bond isomer of lysergic acid) in our lysergic acid-positive transformants indicates that the double-bond isomerization, from position 8,9 (as in agroclavine and elymoclavine) to position 9,10 (as in lysergic acid and derivatives thereof), occurs while substrate is bound to CloA. Moreover, young cultures (<3 days old) yielded lesser quantities of lysergic acid when extracted with methanol but significantly more when extracted with acetic acid-supplemented methanol or with ammonium carbonate. One interpretation of this observation is that the acid or base helped denature CloA, releasing otherwise bound product for detection. While some researchers have hypothesized that isomerization of paspalic acid to lysergic acid happens spontaneously over time (26), the complete lack of paspalic acid in our cultures and the existence of natural variants of *Claviceps paspali* that accumulate large quantities of paspalic acid and lesser quantities of lysergic acid (35) indicate that in a fully functioning lysergic acid pathway the double-bond isomerization is enzymatically catalyzed.

An unexpected and important finding of this study was the accumulation of two novel alkaloids, unknowns A and B, from both transformants but in greater quantities in the *easA-easH* transformants, which accumulated greater concentrations of

TABLE 3 Ergot alkaloids in *A. fumigatus* strains fed 37 nmol of agroclavine and controls

	Amt (nmol/culture) of alkaloid[a]			
Strain/treatment	Agroclavine	Setoclavine[b]	Unknown A	Unknown B
Af 293/agroclavine	22 ± 1 B	2.5 ± 0.1 A	0.56 ± 0.04 A	0.040 ± 0.001 A
NRRL 164/agroclavine	24 ± 2 B	2.1 ± 0.2 AB	ND[c]	ND
easA KO strain/agroclavine	21 ± 0.3 B	1.9 ± 0.08 B	0.22 ± 0.03 B	0.0078 ± 0.0007 B
Medium/agroclavine	31 ± 0.5 A	0.89 ± 0.05 C	ND	ND
Af 293/methanol	ND	ND	ND	ND
NRRL 164/methanol	ND	ND	ND	ND
easA KO strain/methanol	0.041 ± 0.002 C	0.0023 ± 0.0003 D	ND	ND

[a] Data are means for six samples ± SE; values followed by a different letter within a column differ significantly ($\alpha = 0.05$) in a Tukey-Kramer test.
[b] Values calculated from sums of both diastereoisomers.
[c] ND, not detected; limit of detection = 0.01 amol/conidium.

agroclavine and setoclavine. The proposed structure of each unknown, with a prenyl group added in reverse mode to carbon 2 of either agroclavine or setoclavine, comes from five observations. (i) The accumulation of the compounds was dependent on availability of agroclavine provided by either feeding or biosynthesis. (ii) The accumulation of the compounds was restricted to strains of *A. fumigatus* that have a functional copy of the prenyl transferase EasL (also called FgaPT1), which is typically responsible for reverse prenylating fumigaclavine A to fumigaclavine C (Fig. 1) (36). (iii) The molecular weights of the unknowns are consistent with agroclavine and setoclavine with an additional moiety of 68 atomic mass units (amu), which corresponds to the mass of a prenyl group. (iv) The fluorescence properties of each analyte and their long retention times in reverse-phase HPLC are consistent with predicted properties of prenylated forms of agroclavine and setoclavine. (v) The recent discovery of reverse-prenylated versions of festuclavine and fumigaclavine B in *A. fumigatus* (37) indicates that EasL (FgaPT1) accepts other ergot alkaloids as the substrates. While our precursor-feeding and LC/MS data strongly indicate that EasL (FgaPT1) prenylates agroclavine and setoclavine, our proposal that the prenyl group is attached at position 2 in reverse mode can only be hypothesized on the basis of previous mechanistic studies of this enzyme (36) and the consistency of this mode and position of prenylation with other ergot alkaloids of *A. fumigatus* (36, 37). Interestingly, we saw no evidence of prenylation of lysergic acid in *easA-cloA*-expressing *A. fumigatus*, indicating that the carboxylic acid at carbon 17 interfered with enzyme binding or activity. Our data demonstrate that a combinatorial approach based on expression of enzymes from a different lineage of ergot alkaloid producers in *A. fumigatus* can yield novel ergot alkaloids. The biological activities of the novel alkaloids are unknown and will require further study. Based on their structures, however, it is possible that unknowns A and B will have activities similar to those of fumigaclavine C, which has anti-inflammatory activity (38).

In summation, we have reprogrammed *A. fumigatus* to produce lysergic acid by heterologous expression of an isomerase allele of *easA* in a native *easA* knockout strain (to produce agroclavine substrate) concomitant with the expression of the P450 monooxygenase gene *cloA*. This synthetic biology approach presents several intriguing possibilities. The production of lysergic acid in an experimentally tractable and fast-growing organism such as *A. fumigatus* is significant because lysergic acid is used as a base for modification in numerous pharmaceutical products, including the drugs nicergoline, cabergoline, and metergoline. Although *A. fumigatus* is an opportunistic human pathogen, it can be disarmed readily by knocking out confirmed virulence genes (39). Moreover, combinations of enzymes from different lineages gave rise to novel ergot alkaloids, such as the prenylated forms observed in our study. The apparent relaxed specificity of the prenyl transferase EasL may be exploited further for production of other alkaloids. Finally, the expression platform may be conducive to expressing alleles from lineages of fungi producing alternate ergot alkaloids, such as the pharmaceutically relevant dihydroergot alkaloids.

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