Biosynthesis of the Pharmaceutically Important Fungal Ergot Alkaloid Dihydrolysergic Acid Requires a Specialized Allele of *cloA*

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ABSTRACT Ergot alkaloids are specialized fungal metabolites that are important as the bases of several pharmaceuticals. Many ergot alkaloids are derivatives of lysergic acid (LA) and have vasoconstrictive activity, whereas several dihydrolysergic acid (DHLA) derivatives are vasorelaxant. The pathway to LA is established, with the P450 monooxygenase CloA playing a key role in oxidizing its substrate agroclavine to LA. We analyzed the activities of products of *cloA* alleles from different fungi relative to DHLA biosynthesis by expressing them in a mutant of the fungus *Neosartorya fumigata* that accumulates festuclavine, the precursor to DHLA. Transformants expressing CloA from *Epichloë typhina* × *Epichloë festucae*, which oxidizes agroclavine to LA, failed to oxidize festuclavine to DHLA. In substrate feeding experiments, these same transformants oxidized exogenously supplied agroclavine to LA, indicating that a functional CloA was produced. A genomic clone of *cloA* from *Claviceps africana*, a sorghum ergot fungus that produces a DHLA derivative, was cloned and expressed in the festuclavine-accumulating mutant of *N. fumigata*, but several introns in this genomic clone were not processed properly. Expression of a synthetic intron-free version of *C. africana cloA* resulted in the accumulation of DHLA as assessed by fluorescence high-pressure liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS). In substrate feeding experiments, the *C. africana* CloA also accepted agroclavine as the substrate, oxidizing it to LA. The data indicate that a specialized allele of *cloA* is required for DHLA biosynthesis and that the pharmaceutically important compound DHLA can be produced in engineered *N. fumigata*.

IMPORTANCE Ergot alkaloids are fungal metabolites that have impacted humankind historically as poisons and more recently as pharmaceuticals used to treat dementia, migraines, and other disorders. Much is known about the biosynthesis of ergot alkaloids that are derived from lysergic acid (LA), but important questions remain about a parallel pathway to ergot alkaloids derived from dihydrolysergic acid (DHLA). DHLA-derived alkaloids have minor structural differences compared to LA-derived alkaloids but can have very different activities. To understand how DHLA is made, we analyzed activities of a key enzyme in the DHLA pathway and found that it differed from its counterpart in the LA pathway. Our data indicate a critical difference between the two pathways and provide a strategy for producing DHLA by modifying a model fungus. The ability to produce DHLA in a model fungus may facilitate synthesis of DHLA-derived pharmaceuticals.

KEYWORDS ergot alkaloids, lysergic acid, dihydrolysergic acid, P450 monooxygenase

Ergot alkaloids are specialized fungal metabolites that have impacted humankind historically as toxins and more recently as pharmaceuticals used to treat dementia, migraines, hyperprolactinemia, and Parkinson's disease (1–4). Many natural ergot alka-

loids are derivatives of lysergic acid (LA) and have vasoconstrictive activity. Derivatives of dihydrolysergic acid (DHLA), which are rarely encountered naturally, are the basis for many of the commonly prescribed vasorelaxant ergot-alkaloid-derived pharmaceuticals (5–7). LA and its derivatives differ from dihydroergot alkaloids by the presence of a double bond in the fourth (last assembled) or "D" ring of the ergoline nucleus (Fig. 1) and can be converted to their dihydro forms by reduction of that double bond. Only one known naturally occurring fungus produces a DHLA derivative: *Claviceps africana* synthesizes dihydroergosine, a dihydroergopeptine containing DHLA (8). *Claviceps africana* is one of three ergot fungi that parasitize sorghum (*Sorghum bicolor*) (9, 10); the others, *Claviceps sorghicola* and *Claviceps sorghi*, are not known to produce ergot alkaloids. Dihydroergosine is the most complex and abundant dihydroergot alkaloid produced by *C. africana*, but pathway intermediates festuclavine and dihydroelymo-clavine (synonym dihydrolysergol) also accumulate in sorghum infected by this fungus (11, 12). Festuclavine and dihydroelymoclavine must be oxidized to DHLA before incorporation into dihydroergosine (11).

All fungi that make 4-ring ergot alkaloids share five pathway genes (*dmaW*, *easF*, *easC*, *easE*, and *easD*) (Fig. 1) required for assembly of chanoclavine-I aldehyde, the final intermediate prior to formation of the fourth or D ring (7, 13–15). The formation of the D ring may proceed to yield a saturated form, festuclavine, or an unsaturated form, agroclavine, depending on the allele of the gene *easA* present (16–18) (Fig. 1). For example, the allele of *easA* from the fungus *Neosartorya fumigata* (*Aspergillus fumigatus*) reduces the C-8–C-9 double bond in chanoclavine-I aldehyde, allowing rotation of the aldehyde functional group such that it can react with the secondary amine to form an iminium ion that is subsequently reduced by the enzyme EasG (also called FgaFS) to festuclavine (Fig. 1) (19). *Epichloë festucae* (formerly called *Neotyphodium lolii*) has an allele of *easA* that encodes a functionally different enzyme (17). EasA from *E. festucae* appears to only temporarily reduce the C-8–C-9 double bond of chanoclavine-I alde-hyde to allow the isomerization that facilitates the formation of the iminium ion before oxidizing the product to restore the C-8–C-9 double bond as found in agroclavine. LA is produced from agroclavine via primary alcohol (elymoclavine) and aldehyde oxida-tion intermediates by the activity of the P450 monooxygenase named CloA (20, 21). Early labeling studies (11) indicated that DHLA was produced from festuclavine via an analogous set of reactions, but the genetic and enzymatic bases of DHLA production have not been analyzed.

The difference between LA and DHLA is the double bond in the D ring, with associated differences in numbers of hydrogens on C-9 and C-10 (Fig. 1). One possibility is that the only difference between LA and DHLA pathways is formation of agroclavine, in the LA pathway, versus festuclavine, in the DHLA pathway, as intermediates resulting from the differences in activities between the products of the two alleles of *easA*. This scenario would require the four enzymes or enzyme complexes downstream of EasA (EasG, CloA, the Lps1/Lps2 complex, and EasH) in the pathway to ergopeptines or dihydroergopeptines to act on intermediates with either a saturated or unsaturated D ring. This hypothesis is supported by the observations that three of the four enzymes downstream of EasA have been documented to accept saturated and unsaturated forms of their substrates. EasG of *N. fumigata*, which normally acts on the fully reduced iminium ion precursor to festuclavine (Fig. 1), also reduced the unsaturated iminium ion precursor to agroclavine (17–19). Moreover, Lps2 of *Claviceps purpurea* (together with Lps1, as part of the lysergyl peptide synthetase complex) acted on DHLA with approx-imately the same K_m with which it accepted its natural substrate LA (22). Finally, EasH, the last enzyme in the pathway to LA-derived ergopeptines in *C. purpurea*, accepted DHLA-derived dihydroergopeptam substrates as well as its typical LA-derived ergopep-tams (23). The alternative hypothesis is that a specialized festuclavine-oxidizing version of CloA will be required to produce DHLA from festuclavine. In this present study, we address these two alternative hypotheses by expressing alleles of *cloA* from an LA producer and a DHLA producer in a strain of *N. fumigata* that had been modified previously to accumulate festuclavine as its end product by knocking out the gene
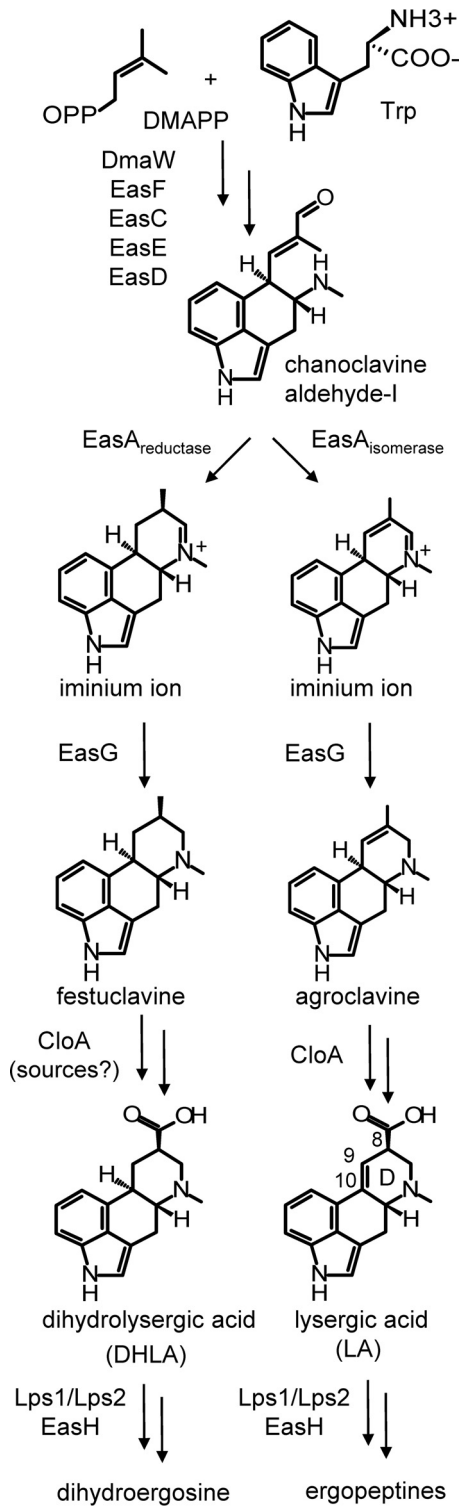
FIG 1 Pathways to lysergic acid and dihydrolysergic acid and their derivatives. Enzymes responsible for catalysis are indicated between intermediates and products. Multiple arrows indicate the omission of intermediates to simplify the figure. Relevant customary ring and carbon labeling is indicated in the lysergic acid structure. DMAPP, dimethylallylpyrophosphate.

easM (24). In *N. fumigata* (which does not have an ortholog of *cloA* in its genome), festuclavine is typically modified by a unique series of enzymatic reactions (initiated by the hydroxylation of festuclavine at C-9 by EasM) to produce a family of ergot alkaloids called fumigaclavines that differ structurally from DHLA and its derivatives
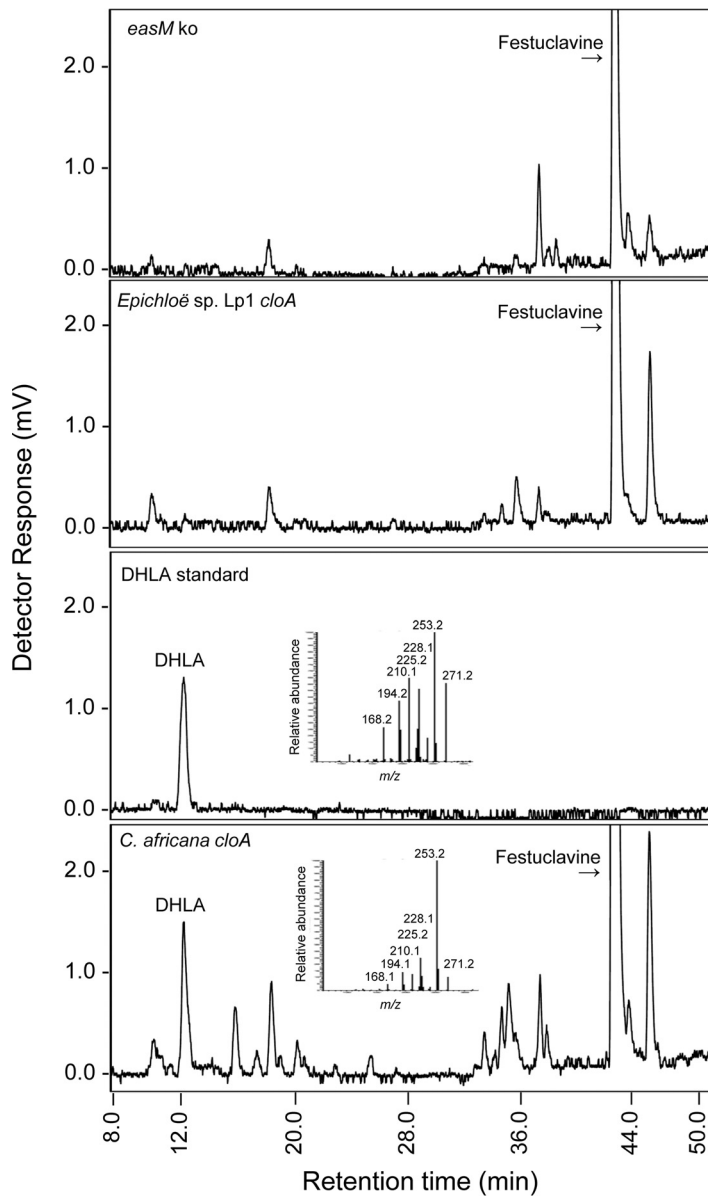
FIG 2 Analysis of ergot alkaloids from transformed strains. HPLC chromatograms of recipient strain (*N. fumigata easM* knockout), transformed strains containing *E. typhina* × *E. festucae* isolate Lp1 *cloA* or synthetic *C. africana cloA*, and DHLA standard. Fluorescence was detected at 372 nm after exciting at 272 nm. Mass spectra of fragmented parent ion of *m/z* 271.2 from DHLA standard and *N. fumigata* expressing synthetic *C. africana cloA* are inserted in relevant chromatograms.

(7, 14, 15, 25). The *easM* step, thus, serves as the branch point between fumiga-clavine and DHLA pathways, and its mutation provides a tool for analysis of DHLA biosynthesis.

RESULTS

The ability of the *Epichloë typhina* × *E. festucae* isolate Lp1 CloA to oxidize festu-clavine to DHLA was evaluated by heterologously expressing its encoding gene in a festuclavine-accumulating mutant of *N. fumigata*. Expression of *E. typhina* × *E. festucae* Lp1 *cloA* under the control of the *N. fumigata easA* promoter did not result in accumulation of DHLA (Fig. 2), and no other novel metabolites were evident in high-pressure liquid chromatography (HPLC) chromatograms. PCR assays indicated that the desired fragments were in the genomes of recipient strains (see Fig. S1 in the
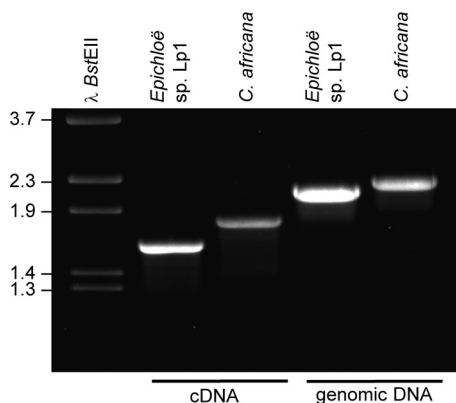
FIG 3 Products derived from cDNA or genomic DNA isolated from *N. fumigata* expressing *E. typhina* × *E. festucae* isolate Lp1 or *C. africana* genomic alleles of *cloA*. Sizes of relevant fragments from BstEII-digested bacteriophage lambda DNA are indicated to the left. Gel was stained with ethidium bromide.

supplemental material), and reverse transcriptase PCR (RT-PCR) assays indicated accumulation of *cloA* mRNA (Fig. 3). Sequencing of the major band of *cloA* cDNA from strains containing *E. typhina* × *E. festucae* Lp1 *cloA* demonstrated that *cloA* transcripts were processed correctly (see Fig. S2 in the supplemental material). Therefore, substrate feeding studies were conducted to test whether functional CloA was present in transformants. Agroclavine supplied exogenously to *E. typhina* × *E. festucae* Lp1 *cloA* transformants was oxidized to LA (Table 1), indicating that functional CloA was present. Controls indicated that accumulation of LA was dependent on the presence of *cloA* in the fungus and exogenously supplied agroclavine. Collectively, the data indicate that expression of *E. typhina* × *E. festucae* Lp1 *cloA* was successful, but festuclavine was not recognized as the substrate by CloA encoded by that particular allele.

A genomic clone of the allele of *cloA* from *C. africana* was tested in a parallel set of experiments. *Claviceps africana* produces dihydroergosine, the dihydroergopeptine derived from DHLA. *Neosartorya fumigata easM* knockout (ko) transformed with *C. africana cloA* under the control of the *N. fumigata easA* promoter failed to oxidize festuclavine to DHLA (data not shown). The *cloA* cDNA from this strain was larger than the expected 1,610-bp fragment (Fig. 3), and when sequenced, it was found to be derived from improperly processed mRNA (Fig. 4; see also Fig. S3 in the supplemental material). Introns 2 and 4 were fully retained, and intron 7 was processed from erroneous 5′ splice junction and 3′ splice junction sequences such that the 5′ portion of the intron was retained, and part of the final exon was removed. Due to the retention of intron 2, the protein translated from this mRNA would have a premature termination codon after 99 amino acids (Fig. 4; see also Fig.

TABLE 1 Oxidation of exogenously fed agroclavine to lysergic acid by *N. fumigata easM* knockout cultures expressing different alleles of *cloA*

Strain[a]	Treatment[b]	Agroclavine (nmol/culture [mean ± SE])	Lysergic acid (nmol/culture [mean ± SE])
easM ko	Agroclavine	12.3 ± 2.3 A[c]	ND[d]
easM ko + *Epichloë* sp. Lp1 *cloA*	Agroclavine	11.0 ± 1.3 A	0.25 ± 0.02 A
easM ko + *C. africana* synthetic *cloA*	Agroclavine	12.4 ± 0.7 A	0.47 ± 0.02 B
Medium	Agroclavine	11.3 ± 2.7 A	ND
easM ko + *Epichloë* sp. Lp1 *cloA*	Methanol	ND	ND
easM ko + *C. africana cloA*	Methanol	ND	ND

[a]$n = 6$.
[b]Agroclavine, 20 nmol.
[c]Means followed by the letter A did not differ significantly in an ANOVA ($P > 0.05$).
[d]ND, not detected; limit of detection = 0.01 pmol/culture.
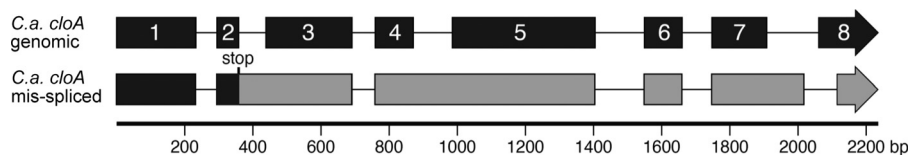
FIG 4 Graphic representation of *C. africana cloA* allele to demonstrate issues with intron processing in *N. fumigata*. Exons in genomic sequence are numbered and colored black. Introns are indicated by thinner lines. In the misspliced transcript, introns 2 and 4 and part of intron 7 are retained in the mRNA as indicated by the thicker, exonic lines. The stop codon resulting from the retention of intron 2 is indicated and exonic sequences downstream of that stop codon are shaded gray.

S4 in the supplemental material), whereas the full-length protein should be 509 amino acids long.

To circumvent the processing issue, a synthetic, intron-free version of *C. africana cloA* was prepared that contained the coding sequences of the gene along with 506 nt of 3'-untranslated DNA fused to the *N. fumigata gpdA* promoter. Codons were optimized for *N. fumigata* translation (see Fig. S5 in the supplemental material), but the amino acid sequence of the gene product was not altered compared to the wild-type version (Fig. S4). Transformation of *N. fumigata easM* ko with the synthetic, coding-sequence-only version of *C. africana cloA* resulted in strains that oxidized festuclavine into DHLA, as evidenced by HPLC with fluorescence detection and mass spectrometry (Fig. 2). In HPLC analyses, a novel peak fluorescing with an excitation wavelength of 272 nm and emission wavelength of 372 coeluted with DHLA standard. In positive mode electrospray ionization liquid chromatography-mass spectrometry (LC-MS), the peak had a parent ion with *m/z* of 271.2 which corresponds to the mass of [DHLA + H]$^+$. When fragmented, the *m/z* 271.2 ion gave rise to fragment ions of the same mass as those liberated by fragmenting the DHLA standard (Fig. 2). Additional novel but minor peaks were detected eluting at approximately 16 and 26 min in the chromatogram of strains expressing the synthetic *C. africana cloA* construct. The identities of these peaks and their potential relationship to DHLA were not investigated further.

Feeding agroclavine, the precursor to LA, to the strain expressing the synthetic *C. africana cloA* resulted in accumulation of LA, indicating that the *C. africana* version of CloA accepts agroclavine as well as festuclavine as the substrate (Table 1). This finding contrasts with the results obtained with CloA from *E. typhina* × *E. festucae* isolate Lp1, which accepted agroclavine but not festuclavine as the substrate. Controls indicated that LA accumulated only when a fungus expressing *cloA* was exposed to exogenously supplied agroclavine.

DISCUSSION

The ergot alkaloid pathway branch to DHLA differs from the branch to LA in two significant ways. First, as previously established (17–20), the reductase form of EasA is required to synthesize the intermediate festuclavine (with its fully reduced D ring) in the DHLA branch, as opposed to production of the unsaturated agroclavine intermediate by the isomerase type of EasA in the LA branch of the pathway (Fig. 1). Second, as shown in this present study, a version of CloA with altered or relaxed substrate specificity is required to oxidize festuclavine to DHLA in the DHLA branch. CloA from the LA-producing fungus *E. typhina* × *E. festucae* isolate Lp1 oxidized agroclavine but not festuclavine as the substrate, whereas the product of the *C. africana* allele oxidized both agroclavine and festuclavine to their respective carboxylic acid forms. The results support the hypothesis that a specialized version of CloA is required to produce DHLA from festuclavine.

Phylogenetic data indicate that the dihydroergot alkaloid pathway in *C. africana* evolved in a lineage of fungi that produced LA-derived ergot alkaloids (13). Two novel traits were required in the evolution of the DHLA branch of the ergot alkaloid pathway, alternative alleles of *easA* and *cloA*. The change in alleles of *easA* leading to production

of festuclavine as opposed to agroclavine is clearly a prerequisite to DHLA synthesis, but the necessity for a novel allele of *cloA* for DHLA biosynthesis was not evident *a priori*. Reasons for initially hypothesizing that enzymes downstream of festuclavine and agroclavine would accept either of those intermediates as the substrates included previous studies indicating that the three downstream enzymes (or enzyme complexes) other than CloA that interact with the relevant intermediates (EasG, Lps2 as part of the lysergyl peptide synthetase complex, and EasH) accepted saturated (festuclavine-derived) and unsaturated (agroclavine-derived) substrates (17, 18, 22, 23). Hypothetically, the failure of the *E. typhina* × *E. festucae* isolate Lp1 version of CloA to recognize festuclavine as the substrate may result from steric hindrances due to additional hydrogens on both carbon 9 and carbon 10. The changes in the *C. africana* version of CloA that allowed festuclavine to serve as the substrate still allowed it to accept agroclavine.

One can envision the novel alleles of *easA* and *cloA* arising in either of two possible sequences. If evolution of the version of CloA with relaxed or altered specificity occurred first, the fungus probably would have been able to continue to produce LA-derived ergot alkaloids from the agroclavine substrate, since in this current study, *C. africana* CloA oxidized agroclavine to LA. When in this lineage *easA* mutated to the reductase allele, the fungus would then have been preadapted to accept festuclavine as the substrate and make DHLA and DHLA-derivatives. Alternatively, if the version of *easA* changed first, festuclavine would have accumulated and there would have then been pressure on the fungus to evolve an allele of *cloA* that encoded a product capable of accepting festuclavine as the substrate. The events in either scenario fit well with the screening hypothesis of Firn and Jones (26), since the events increase chemical diversity and form a new pathway branch.

The inability of *N. fumigata* to process the *C. africana cloA* introns properly was unexpected. Retained intron 2 had a 3′ splice site of AAG, which differs from the more common YAG sequence typically found in fungi (27); however, intron 1 also ended with AAG and was processed properly. The introns that were retained had a predominance of adenosine homopolymers. Retained intron 2 contained a run of 25 consecutive A residues immediately before and into the 3′ splice site sequence. Retained intron 4 had a series of 9 consecutive A residues between the predicted branch site and the 3′ splice site. Retained intron 7 had a string of 69 nucleotides in which 60 A residues occurred between the 5′ splice site and the presumed branch site. It should be noted, however, that intron 5, which was processed properly, contained a string of 54 nucleotides, of which 42 were A residues between its 5′ splice site and the presumed branch site.

In addition to removal of introns, the synthetic *C. africana cloA* allele differed from the native allele in two other ways, (i) a *gpdA* promoter from *N. fumigata* was used on the synthetic allele, whereas an *N. fumigata easA* promoter drove expression of the native allele, and (ii) codon usage was altered in the synthetic allele to conform with preferences observed in *N. fumigata* (28) without altering the protein sequence. These changes might affect the quantity of DHLA produced; however; the qualitative presence/absence of DHLA observed almost certainly resulted from the production of a full-length protein. RT-PCR analyses indicated that abundant mRNA was produced from the original *C. africana cloA* genomic construct, although that mRNA was not properly processed. Another factor that potentially affected relative expression of the different constructs in this study was that their integration in the genome was likely random. Fifteen to 20 transformants for each construct were thus analyzed to control any position effects on expression via randomization.

Whereas our results show accumulation of DHLA in *N. fumigata* transformants expressing the intron-free allele of *C. africana cloA*, relatively large quantities of festuclavine were retained, as evidenced by relative peak height in HPLC chromatograms. Determination of the reason for this relatively poor turnover was not the purpose of this current study, but potential reasons include poor expression or stability of the introduced CloA or differences in localization of CloA and its substrate in the

TABLE 2 PCR primers and products

Primer combination	Primer sequences (5′→3′)	Product	Amplicon length(s) (bp)
1	CATCGAATTCGTTGACATTGCTTCTAATCCACC, GACAGCGGCCGCAACAAGCGATAAGCGTTAG	*Epichloë* sp. Lp1 *cloA* + *N. fumigata* promoter	3,526
2	GTACTTGGTGGATTAGAAGCAATGTCGCAACTATGGCTATACAAGGC, GACAGCGGCCGCGTGTTCACGCCACGTCACC	*C. africana cloA* with promoter extension[a]	2,569
3	CATCGAATTCTGCCTACTCTATAGAAGATGGATC, GCCTTGTATAGCCATAGTTGCGACATTGCTTCTAATCCACCAAGTAC	Promoter with *C. africana cloA* extension	811
4	CATCGAATTCTGCCTACTCTATAGAAGATGGATC, GACAGCGGCCGCGTGTTCACGCCACGTCACC	Promoter/*C. africana cloA* fusion construct	3,334
5	CTATAGAGTAGGCACTCCGCAC, CTCCAAGTCAAGATACATCCCTC	*Epichloë* sp. Lp1 *cloA*	1,610 (cDNA), 2,178 (gDNA[b])
6	CTATAGAGTAGGCACTCCGCAC, GCGAGATAAATGAGAAGAACCATTG	*C. africana cloA*	1,861 (cDNA), 2,318 (gDNA)

[a]Extension refers to incorporation of an additional 21 or 23 nucleotide (nt) at the 5′ end of a primer to add sequences that will facilitate a later fusion PCR.
[b]Genomic DNA.

heterologous host, *N. fumigata*. Substrate feeding studies with agroclavine produced conversions comparable to those observed in a previous study in which native *N. fumigata* enzymes prenylated when exogenously fed agroclavine substrate (21). This observation indicates that neither protein expression nor stability was a major factor. Issues related to localization or other potential reasons for the modest turnover will need to be addressed in order for a DHLA-producing strain to have practical value. An ability to produce abundant DHLA in a manipulable fungus like *N. fumigata* might provide opportunities for improved synthesis of DHLA-derived compounds.

MATERIALS AND METHODS

Modification of fungi. All *cloA* constructs were expressed in a mutant of *N. fumigata* (*Aspergillus fumigatus*) Af293 in which the *easM* gene had been knocked out (*N. fumigata easM* ko) to cause the strain to accumulate festuclavine (24), a precursor to DHLA (11). Since the *N. fumigata easM* ko strain is resistant to hygromycin, all *cloA* expression constructs were prepared in pBCphleo (29; Fungal Genetics Stock Center, Manhattan, KS), which confers resistance to phleomycin.

Two *cloA* expression constructs were prepared by PCR from genomic DNA in a series of reactions catalyzed by Phusion DNA polymerase (Thermo Fisher, Waltham, MA). In general, PCR analysis was conducted as follows: an initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 15 s, primer annealing at 62°C for 15 s, and primer extension at 72°C for 60 s. Variations in annealing temperature or extension time are noted below for particular primer combinations. The *E. typhina* × *E. festucae* isolate Lp1 (30) *cloA* construct consisted of the *easA* promoter of *N. fumigata* (operationally defined as the 787 bp immediately upstream of the initiation codon) fused to the genomic *E. typhina* × *E. festucae* Lp1 *cloA* coding sequences along with 622 nt of 3′-untranslated sequences. The promoter/coding sequence fragment was PCR amplified from the *E. typhina* × *E. festucae* isolate Lp1 *easA-cloA* strain described in Robinson and Panaccione (21) in a reaction with primer combination 1 (Table 2). PCR conditions were as described above, but the annealing temperature was 67°C. The PCR product was cleaned with the DNA Clean and Concentrator-5 kit (Zymo Research, Irvine, CA), digested with EcoRI and NotI (New England BioLabs, Ipswich, MA) (sites for which were included in primers), and ligated into EcoRI/NotI-digested pBCphleo. To prepare the *C. africana* isolate Cla9 (10) genomic *cloA* construct, coding sequences of *cloA* and 300 bp of 3′ flanking sequences were PCR amplified from primer combination 2 (Table 2). Primers were designed based on the sequence of a contig obtained from a draft genome sequence of *C. africana* isolate Cla 9, which has been deposited in GenBank as accession number KY677717. The *N. fumigata easA* promoter (amplified with primer combination 3; Table 2) was attached to coding sequence fragments in a fusion PCR with approximately equimolar concentrations of *C. africana cloA* coding sequences and *N. fumigata easA* promoter fragments as the template and primer combination 4 (Table 2). Reaction conditions were as described above but with an extension time of 75 s. The fusion PCR product was cleaned as described above, digested with EcoRI and NotI (sites included in primers for fusion PCR), and ligated into EcoRI/NotI-digested pBCphleo. Sequence integrity of constructs was confirmed by Sanger methodology at Eurofins Genomics (Louisville, KY).

The coding sequence of synthetic *cloA* construct (GenBank accession no. KY681814) was derived from the *C. africana* allele by deleting introns (as marked in Fig. S3 in the supplemental material) and changing nucleotides to favor codons considered optimal for *N. fumigata* in the analyses of Iriarte et al. (28) without altering amino acid sequence of the product (see Fig. S4). The synthetic coding sequences were placed under the control of the promoter of the *N. fumigata* glyceraldehyde-3-phosphate dehydrogenase gene, *gpdA* (GenBank accession no. AM999768), operationally defined as the 962 bp immediately preceding the initiation codon. The synthetic construct also contained 506 bp of 3′-untranslated

sequence as found immediately downstream of the stop codon of the native *C. africana cloA* allele. The construct was prepared and sequenced by GenScript (Piscataway, NJ). The expression construct was removed from its original pUC19 vector by digestion with EcoRI and ligated into EcoRI-digested pBCphleo for transformation into *N. fumigata easM* ko.

The *N. fumigata easM* ko strain was transformed essentially as described previously (18, 24). Spheroplasts were prepared with cell wall-degrading enzymes Vinotaste (Gusmer Enterprises, Mountainside, NJ) and lysing enzyme from *Trichoderma harzianum* (Sigma Chemical, St. Louis, MO), and DNA was introduced via the polyethylene glycol–CaCl$_2$ method. Transformants were selected on complete regeneration medium containing phleomycin (InvivoGen, San Diego, CA) at 400 μg/ml and purified to nuclear homogeneity by culturing from single conidia. Fifteen to 20 transformants obtained with each construct were analyzed by HPLC (described below) to account for potential variation associated with random integration of the *cloA* expression constructs.

Analysis of transformants. DNA was isolated from transformants with a GeneClean kit (MP Biomedical, Santa Ana, CA) and analyzed for the presence of introduced fragments by PCR as described above with primer combination 1 (Table 2). RNA was isolated from 100 mg of 2-day-old surface cultures of native and transformed strains of *N. fumigata* grown on malt extract broth by application of the Qiagen RNeasy Plant kit. Polyadenylated mRNA was primed with oligo(dT) and reverse transcribed into cDNA in a reaction with Superscript IV reverse transcriptase (Thermo Fisher). Resulting cDNA was amplified with primer combination 5 for *E. typhina* $\times$ *E. festucae* Lp1 or primer combination 6 for *C. africana* (Table 2). PCR conditions were as described above but with an annealing temperature of 58°C and an extension time of 45 s. PCR products were sequenced by Sanger methodology at Eurofins Genomics (Louisville, KY).

Ergot alkaloids were extracted from conidiating cultures of *N. fumigata* in HPLC-grade methanol and analyzed by reverse-phase HPLC as described previously (31). Briefly, the column was a 150 $\times$ 4.6 mm Prodigy ODS3 with 5-μm particle size (Phenomenex, Torrance, CA), and the mobile phase was a binary, multilinear gradient from 5% acetonitrile (Fisher, Pittsburgh, PA) + 95% 50 mM ammonium acetate to 75% acetonitrile + 25% 50 mM aqueous ammonium acetate. Analytes were detected by fluorescence at two wavelengths: excitation at 272 nm with emission at 372 nm for ergot alkaloids lacking a C-9–C-10 unsaturation in the D ring (such as festuclavine and DHLA) (Fig. 1), and 310-nm excitation with 410-nm emission for ergot alkaloids containing a C-9–C-10 double bond in the D ring (such as LA). DHLA standard was prepared by hydrolyzing the peptide bonds in dihydroergocristine (Sigma) in 1.2 M NaOH at 75°C for 6 h. The solution was then brought to neutral pH with the addition of 0.5 volume of 2.4 M acetic acid. Standard prepared in this manner was analyzed for fluorescence properties characteristic of DHLA (excitation at 272 nm with emission at 372 nm) and by high-resolution mass spectrometry (calculated *m/z*, 271.1441; measured *m/z*, 271.1440; deviation, 0.37 ppm). Electrospray ionization liquid chromatography–mass spectrometry (LC-MS) was performed in positive mode on a Thermo LCQ Deca XP Plus equipped with a Surveyor HPLC as described by Ryan et al. (32). DHLA standard was analyzed on a Thermo Q Exactive mass spectrometer as described by Robinson and Panaccione (21).

Production of LA through a substrate feeding approach. Substrate feeding studies were conducted as described by Robinson and Panaccione (21) with minor modifications. Briefly, six replicate cultures of each treatment or control group were grown from 60,000 conidia in 200 μl malt extract broth supplemented with 20 nmol agroclavine in 1 μl of methanol or, in controls, with 1 μl of methanol. After 1 week, ergot alkaloids were extracted by the addition of 300 μl of methanol and analyzed by HPLC with fluorescence detection as described above. Agroclavine was quantified by comparing peak areas to standard curve prepared from known quantities of agroclavine (Fisher), whereas LA was quantified relative to a standard curve prepared from ergonovine (Sigma). Quantities of alkaloids across treatments and controls were compared by analysis of variance (ANOVA). All statistical analyses were performed with JMP software (SAS, Cary, NC).

Accession number(s). The sequences described were deposited in GenBank under the accession numbers KY677717 and KY681814.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM .00805-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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