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Comparison of Ergot Alkaloid Biosynthesis Gene Clusters in *Claviceps* Species Indicates Loss of Late Pathway Steps in Evolution of *C. fusiformis*▽

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The grass parasites *Claviceps purpurea* and *Claviceps fusiformis* produce ergot alkaloids (EA) in planta and in submerged culture. Whereas EA synthesis (EAS) in *C. purpurea* proceeds via clavine intermediates to lysergic acid and the complex ergopeptines, *C. fusiformis* produces only agroclavine and elymoclavine. In *C. purpurea* the EAS gene (*EAS*) cluster includes *dmaW* (encoding the first pathway step), *cloA* (elymoclavine oxidation to lysergic acid), and the *lpsA*/*lpsB* genes (ergopeptine formation). We analyzed the corresponding *C. fusiformis EAS* cluster to investigate the evolutionary basis for chemotypic differences between the *Claviceps* species. Other than three peptide synthetase genes (*lpsC* and the tandem paralogues *lpsA1* and *lpsA2*), homologues of all *C. purpurea EAS* genes were identified in *C. fusiformis*, including homologues of *lpsB* and *cloA*, which in *C. purpurea* encode enzymes for steps after clavine synthesis. Rearrangement of the cluster was evident around *lpsB*, which is truncated in *C. fusiformis*. This and several frameshift mutations render Cf*lpsB* a pseudogene (Cf*lpsB*$^{\Psi}$). No obvious inactivating mutation was identified in Cf*cloA*. All *C. fusiformis EAS* genes, including Cf*lpsB*$^{\Psi}$ and Cf*cloA*, were expressed in culture. Cross-complementation analyses demonstrated that Cf*cloA* and Cf*lpsB*$^{\Psi}$ were expressed in *C. purpurea* but did not encode functional enzymes. In contrast, Cp*cloA* catalyzed lysergic acid biosynthesis in *C. fusiformis*, indicating that *C. fusiformis* terminates its EAS pathway at elymoclavine because the *cloA* gene product is inactive. We propose that the *C. fusiformis EAS* cluster evolved from a more complete cluster by loss of some *lps* genes and by rearrangements and mutations inactivating *lpsB* and *cloA*.

Numerous phytopathogenic fungi belonging to the genus *Claviceps* produce ergot alkaloids (EA), which are tri- or tetracyclic derivatives of prenylated tryptophan. Some EA show structural similarity to the neurotransmitters serotonin and dopamine and have affinity to the cognate receptors in the central nervous system (1, 12). Therefore, EA have long been used for treatment of a variety of disorders of the central and peripheral nervous systems (22).

EA for pharmaceutical use are produced using *Claviceps purpurea*, a ubiquitous fungus that infects nearly 600 grasses and cereals and causes ergot disease (2). These alkaloids accumulate in sclerotia ("ergots"), the dense fungal resting structures that replace host seeds. The biochemistry of EA formation in *C. purpurea*, *Claviceps fusiformis*, and other fungi has been studied in detail (for reviews, see references 6 and 17). The first reaction of the pathway is prenylation of tryptophan with formation of dimethylallyltryptophan (Fig. 1). Biosynthesis proceeds to production of clavine alkaloids such as agroclavine and elymoclavine, the end product in *C. fusiformis*. In *C. purpurea* the pathway continues to lysergic acid, and the end products of the pathway are lysergic acid amides, including the complex ergopeptines.

Roles for several genes of the EA synthesis (EAS) gene cluster (*EAS* cluster) in *C. purpurea* have been characterized to date. The gene encoding the enzyme catalyzing the first step (tryptophan aromatic prenylation) is *dmaW*. The *cloA* gene product converts elymoclavine to paspalic acid, which undergoes a spontaneous isomerization to lysergic acid. Lysergyl peptide synthetase 1 (a nonribosomal peptide synthetase [NRPS]) is a complex consisting of the products of *lpsB* and one of the two *lpsA* paralogues, *lpsA1* or *lpsA2*. For example, the complex consisting of the *lpsA1* and *lpsB* gene products catalyzes formation of ergotamam, which is then oxidized (presumably by another enzyme) to ergotamine (3). These genes have been functionally analyzed by heterologous expression or gene replacement approaches (3, 8, 21). The cluster also contains genes predicted to encode oxidoreductases, a catalase, and an additional NRPS, LpsC, the function of which is still not known (7, 23).

Whereas *C. purpurea* produces lysergic acid and its complex derivatives, the EAS pathway in *C. fusiformis* terminates at elymoclavine. These two fungi have similar life cycles, but the host range of *C. fusiformis* is limited to pearl millet (*Pennisetum glaucum*) and buffel grass (*Pennisetum ciliare*), whereas *C. purpurea* infects temperate grasses (subfamily Pooideae). Here we describe a comparative sequence analysis of *EAS* cluster genes in these two fungi, as well as expression studies, to determine the genetic basis for differences in their EA profiles.

MATERIALS AND METHODS

Strains and culture conditions. *C. purpurea* strain P1 (= ATCC 20102), which produces mainly ergotamine along with low levels of ergocryptine, was described previously (10, 23), as were the standard media and culture conditions (23). *C. fusiformis* strain SD58 (= ATCC 26245), which produces mainly agroclavine and elymoclavine, and its culture growth conditions have been described previously (21). For alkaloid production and for RNA expression studies, *C. purpurea* was cultivated in T25N medium (23) and *C. fusiformis* was cultivated in T2 medium

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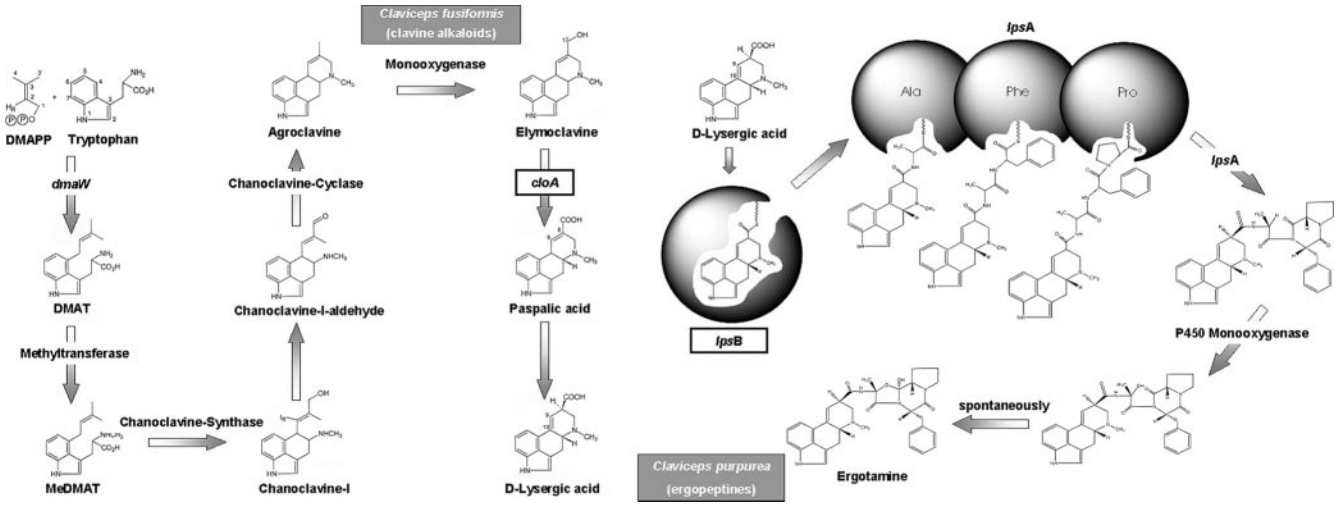

FIG. 1. Diagrams of EA biosynthesis in *C. fusiformis* (end product, elymoclavine) and *C. purpurea* (ergopeptides). Genes of interest are enclosed in boxes. DMAPP, dimethylallylpyrophosphate; DMAT, dimethylallyltryptophan; MeDMAT, methyl dimethylallyltryptophan. (Modified from reference 7 with permission.)

[100 g/liter saccharose, 10 g/liter L-asparagine, 1 g/liter $Ca(NO_3)_2$, 0.1 g/liter yeast extract, 0.25 g/liter $MgSO_4 \cdot 7H_2O$, 0.15 g/liter $ZnSO_4 \cdot 7H_2O$] with low (0.5 g/liter KH_2PO_4) and high (2.0 g/liter KH_2PO_4) levels of phosphates.

Molecular biology techniques. Standard cloning and DNA analysis techniques were performed as described by Sambrook et al. (16). The *Escherichia coli* strain used for cloning (by using plasmids pUC19 [Fermentas, St. Leon-Rot, Germany] and pCR2.1-TOPO [Invitrogen, Karlsruhe, Germany]) and propagation of clones was TOP10F′ (Invitrogen, Karlsruhe, Germany). Extraction of genomic DNA, Southern and Northern blot analyses, and DNA sequencing were performed as described previously (13). Genomic Southern analyses of *dmaW* and *lpsA* were conducted as described by Wang et al. (24) and Panaccione et al. (14), respectively. For sequence comparisons and multiple-sequence alignment DNA STAR was used. For PCR analysis, BioTherm (Genecraft, Lüdinghausen, Germany) polymerase was used according to the manufacturer's instructions.

Cosmid clones, transposon insertion library, and sequencing. A cosmid clone of *C. fusiformis*, designated Cf26E11, was previously identified as a clone containing *dmaW* (21). A library of transposon insertions in Cf26E11 was created using a HyperMu <KAN1> insertion kit (EPICENTRE Biotechnologies, Madison, WI) according to the manufacturer's instructions. Positive clones were selected on LB agar with kanamycin and ampicillin. The cosmid DNAs were isolated from bacterial cultures with Perfectprep BAC 96 (Eppendorf, Westbury, NY). DNA sequencing from both ends of each HyperMu insert was performed at the University of Kentucky Advanced Genetic Technologies Center, using a BigDye Terminator v3.1 cycle sequencing kit, a GeneAmp 9700 PCR system thermocycler, and a 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). Sequencing was conducted on eight 96-well plates containing cosmids with independent HyperMu insertions.

Sequences were viewed and assembled using the Phred-Phrap package and were viewed with Consed for Linux Systems (D. Gordon, University of Washington). Coding sequences of *C. fusiformis EAS* genes were identified using the BLASTX resource of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Exons were determined by combining results of three NCBI BLAST alignments (BLASTX, TBLASTX, and BLASTN) and also by using gene predictions obtained with Fgenesh (http://sun1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind) using *Aspergillus* spp., *Magnaporthe oryzae* (*Magnaporthe grisea*), and *Neurospora crassa* as reference fungi. Protein family signatures were identified with InterProScan (http://www.ebi.ac.uk/InterProScan/) and Prosite (http://www.expasy.ch/prosite/).

RNA isolation and reverse transcription PCR (RT-PCR). Total RNA extraction from 7- to 14-day-old mycelia and DNase treatment were performed as described previously (18) using the RNAgents total RNA isolation system (Promega, Mannheim, Germany). Concentrations of purified RNA were determined using a BioPhotometer (Eppendorf, Hamburg, Germany), and RNA integrity was examined by electrophoresis in 1% agarose gels. For gene expression studies, total RNA was isolated from EA-inducing and -suppressing cultures, and the derived cDNA was synthesized and amplified with the SuperScript-II reverse transcriptase (Invitrogen, Karlsruhe, Germany) as described previously (20). The primers used for sequencing reactions are summarized in Table 1. For expression studies, sequencing of *EAS* genes from *C. fusiformis* cDNA was performed with primers to obtain products that span at least one intron of each gene. Splicing of the eighth intron of Cf*cloA* was examined by PCR and sequencing with primers MO3 and MO2.

Construction of complementation vectors and transformation of *C. purpurea* and *C. fusiformis*. Clones for complementation of *C. purpurea* deletion mutants were constructed by ligating restriction fragments from Cf26E11 into pUC19 (Fermentas, St. Leon-Rot, Germany). One clone contained Cf*cloA* in a 5.8-kb XbaI fragment, and another clone contained Cf*cloA* in a 3.9-kb EcoRI fragment. Transformation of protoplasts was performed as described previously (3), using the vector pAN7.1_UM, which includes a hygromycin resistance gene, as the covector for transformant selection. Plasmid cP450-1 (8), containing Cp*cloA* and a hygromycin resistance gene, was used for transformation of *C. fusiformis*.

PCR. To test for integration of the Cf*lpsB* pseudogene (Cf*lpsB*$^{\Psi}$), PCR was performed with primers cf*lpsB*_f and cf*lpsB*_r. To test for integration of Cf*cloA*, PCR was performed with primers cf*cloA*_f and cf*cloA*_r.

The primers used to test for integration of the complete DNA segment, including the *cloA* promoter and the left portion of the gene, were HI-LF-P450-1 and HI-WT-P450-1. Integration of the right portion of the gene and its terminator was tested by PCR with primers HI-RF-P450-1 and UR450.

For details concerning the sequences of the primers see Table 1.

Extraction and analyses of ergot alkaloids. For alkaloid extraction and determination, the pH of cultures was adjusted to 11 with concentrated aqueous ammonium hydroxide, the cultures were extracted three times with chloroform, and, after concentration, the resulting liquid was applied to thin-layer chromatography (TLC) plates (Silica Gel 60; Merck, Darmstadt, Germany). Alkaloids were identified by comparison with corresponding standards. After separation in CH_3CN-isopropanol-H_2O (125:28:15, vol/vol/vol), TLC plates were sprayed with van Urk's reagent for alkaloid visualization. The standards used were 1 mg/ml ergotamine (Sigma, Taufkirchen, Germany), 1 mg/ml ergocryptine (Sigma, Taufkirchen, Germany), 1 mg/ml ergocristine (Sigma, Taufkirchen, Germany), 1 mg/ml agroclavine, and 1 mg/ml D-lysergic acid (Sandoz AG, Basel, Switzerland).

High-performance liquid chromatography (HPLC) separation was carried out using a LiChrospher 100 RP-18 column (5 ml; 250 by 4 mm; Merck/Hitachi, Darmstadt, Germany) operated at 25°C and a flow rate of 1 ml/min. Compounds were eluted using a 1:1 mixture of 10 mM $(NH_4)_2CO_3$ and CH_3CN for 20 to 40 min and were detected with an L-7400 UV detector. The EA standards used were those described above.

Mass spectroscopy analyses. The HPLC-electrospray ionization-mass spectroscopy system consisted of an analytical liquid chromatography system (1100 series; Agilent Technologies Deutschland GmbH, Böblingen, Germany) coupled to a QTrap2000 with a TurboIonSpray source (Applied Biosystems, Darmstadt, Germany). Separation was performed on a Luna 5m C_{18} (2) column (100A; 100 by 4.6 mm; Phenomenex, Aschaffenburg, Germany) with a flow rate of 1,500 μl/min, and 400 μl/min was directed into a mass spectrometer.

TABLE 1. Primers used for RT-PCR and sequencing reactions

Use	Forward primer		Reverse primer	
	Designation	Sequence (5′–3′)	Designation	Sequence (5′–3′)
C. fusiformis EAS gene expression	CfdmaW_1d	ACACGGATGGTATGAGATGGC	Cf_dmaW_3u	CTACGTCAGGGACAAGTCAC
	Cf_easG_d	GTTTGCACGCACGGGAATAC	Cf_easG_u	AGGCAGTGGCAAGACTGCTG
	Cf_easF_d	AACGAAGGACTGGACCACTG	Cf_easF_u	GTGTTTCGCTGATCCATCGT
	Cf_easE_d	AAGTATATGCTCATGGTGTC	Cf_easE_u	CATATCATTGAAGTGCTGCA
	Cf_easD_d	TTGGCTCGCGGTACAGCTTC	Cf_easD_u	GACGATTCTCGAGGAAGACG
	Cf_easC_d	GTCAACTTGCCTACACATCG	Cf_easC_u	GGACCTCGTACATGCTCAAC
	Cf_cloA_d	AATCATGGCGTCCAGTCCAC	Cf_cloA_u	CTCTGGCTATACCAAGCTCT
	Cf_lpsB_d	CTGTTCACGAGAGCATCGCG	Cf_lpsB_u	CGAAACTACCATCTCAGAGC
	MO3	TCAGGTCCATCGATCAGCCAGTAT	MO2	TTTTCATCCTCTTCGCAACATTCC
C. fusiformis lpsB integration test	Cf_*lpsB*_f	TGCGGCGAGGAACGAAGAATGG	Cf_*lpsB*_r	GAGACTGCGGCGGGAAGGCTAATC
C. fusiformis cloA integration test	Cf_*cloA*_f	CCTCCGAGCAGCCTTACTTTT	Cf_*cloA*_r	CTAGGCGCGTTGACCGACTTGT
	HI-LF-P450–1	CCCCTAGGACATTGATCGGAGA	HI-WT-P450–1	AAGGGGCATTACTATAGGTTCTTTC
	UR450	ATCGATCTGCCCTTCGCTTTCTCGTT	HI-RF-P450–1	CGCTTGAACCCGCTATTACGAG

RESULTS AND DISCUSSION

Comparison of the *EAS* cluster sequences of *C. purpurea* and *C. fusiformis*. The *EAS* cluster sequence of *C. purpurea* P1 comprises 68.5 kb and includes 13 genes that are coordinately induced in EA production conditions (7). A clone (cosmid Cf26E11) of the homologous genomic region in *C. fusiformis* SD58 was previously obtained by screening a cosmid library with *dmaW* cDNA as a probe (21). We sequenced this cosmid and obtained a 35.4-kb contig, in which a 19.6-kb region contains nine homologues of the *C. purpurea EAS* cluster genes (Fig. 2). Corresponding to the orientation of the *C. purpurea* cluster, *dmaW* represents the right border of the sequenced *C. fusiformis EAS* cluster; the left border was flanked by a predicted aminopeptidase family gene (Fig. 2), to the left of which 15 hypothetical protein genes were identified. The corresponding location of the *C. purpurea* cluster was not homologous to the aminopeptidase gene or these hypothetical protein genes and instead contained (from right to left) another putative NRPS gene (*lpsC*), a gene encoding another hypothetical protein (*orfD*), and a putative amino acid biosynthesis gene (*imd*) (7).

RT-PCR indicated that all genes of the *C. fusiformis* cluster were expressed in EA-producing cultures (data not shown). The expressed genes included the homologue of Cp*cloA*, which encodes a cytochrome P450 monooxygenase necessary for synthesis of lysergic acid from elymoclavine. Also expressed was the homologue of Cp*lpsB*, which encodes the smaller subunit of the large NRPS, lysergyl peptide synthetase (23).

Considering the fact that *C. fusiformis* accumulates elymoclavine and not lysergic acid or its amides, there is no obvious role for *cloA* or *lpsB* in this species, and the possibility was considered that these genes are pseudogenes. A comparison of the amino acid sequences predicted for the *cloA* homologues in *C. purpurea* and *C. fusiformis* revealed 66.4% identity. This level of divergence was comparable to that between the functional homologues Cf*dmaW* and Cp*dmaW*. Within the heme-binding domain the level of identity was 80%. Therefore, there was no reason to expect that the gene has been inactive for a long evolutionary time. Furthermore, it was not apparent that any of the differences in Cf*cloA* might affect the function of the protein product. Also, a comparison with the amino acid sequence derived from the putative orthologue from *Epichloë festucae* did not give any hint of a functional defect (data not shown). Therefore, we investigated whether Cf*cloA* mRNA may be incorrectly processed. The gene sequence indicated the same exon-intron structure as Cp*cloA*, although a potentially significant change was observed in the last intron, where Cf*cloA* had a predicted 5′ GC splice junction, whereas Cp*cloA*

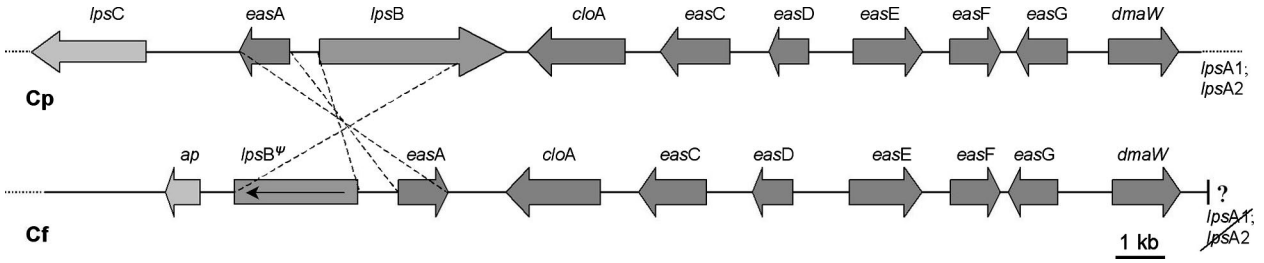

FIG. 2. Schematic comparison of the *EAS* clusters of *C. purpurea* (top) and *C. fusiformis* (bottom). Homologous genes in the two species are indicated by dark gray arrows, whereas genes identified in only one species are indicated by light gray arrows. The arrows indicate the orientation of transcription. The genes, as well as the intergenic regions, are drawn to scale. Rearrangement in the locus, indicated by dashed lines, is associated with truncation of *lpsB* in *C. fusiformis*. *ap* is a putative aminopeptidase gene adjacent to the *C. fusiformis EAS* cluster. The accession no. for the *C. fusiformis* cluster sequence is EU006773.

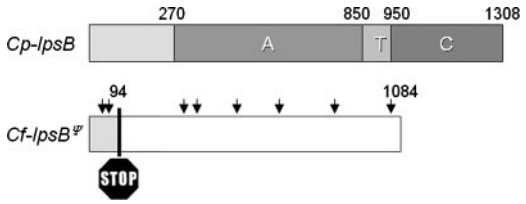

FIG. 3. Schematic comparison of Cp*lpsB* and its homologue Cf*lpsB*$^{\Psi}$. Adenylation (A), thiolation (T), and condensation (C) domains are indicated by shaded boxes, and the numbers indicate the domain borders (amino acids) for Cp*lpsB*. The arrows indicate frameshift mutations in Cf*lpsB*$^{\Psi}$ resulting in a premature stop codon at position 94. The open box indicates the portion of Cf*lpsB*$^{\Psi}$ downstream of the stop codon that aligns with Cp*lpsB*.

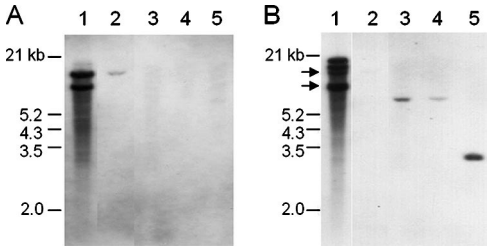

FIG. 4. Southern blot analysis of genomic DNA from several EA-producing fungi. The membrane was first hybridized to a probe from Cp*dmaW* (A) and then stripped and hybridized to probe from Cp*lpsA* (B). Samples (4 μg) of total fungal DNA were digested with EcoRI to completion, fractionated by electrophoresis in a 0.9% agarose gel, and transferred to a nylon membrane. The lanes contained DNA from *C. purpurea* P1 (lane 1), *C. fusiformis* SD58 (lane 2), *Neotyphodium coenophialum* ATCC 90664 (lane 3), *N. lolii* × *Epichloë typhina* Lp1 (lane 4), and *B. obtecta* B249 (lane 5). The arrows in panel B indicate the positions of a residual signal from the Cp*dmaW* probe; the lower band (8.3 kb) was close to the position of an authentic *lpsA* signal.

had the more common GT junction. RT-PCR analysis and sequence analysis of the products indicated that the expressed Cf*cloA* transcript was nevertheless properly spliced (data not shown).

In the case of Cf*lpsB*$^{\Psi}$, numerous indel mutations shifted the reading frame throughout the coding sequence (Fig. 3). An in silico correction of these frameshifts gave a predicted product with 61.9% identity to the product of Cp*lpsB* (Fig. 3). However, this region of similarity extends only to codon 1084 of the 1,308 codons of Cp*lpsB*, and there is no recognizable match between the remaining 3′ portion of the *C. purpurea* gene in the *C. fusiformis* locus. Thus, Cf*lpsB*$^{\Psi}$ lacks coding sequence for most of the last (condensation) domain of LPS2.

The critical changes found in the first exon of Cf*lpsB*$^{\Psi}$ included two deletions, 4 bp each, which shifted the reading frame to a reading frame ending with a stop at codon position 94 (Fig. 3). Another stop codon in this reading frame was at position 117. Therefore, only a short, nonfunctional peptide was expected as a product of this pseudogene. The insert in cosmid clone Cf26E11 contained no apparent fungal genes downstream of *dmaW*, whereas the corresponding region of the known *EAS* cluster in *C. purpurea* has genes for a putative nonheme iron oxygenase (*easH*) and the paralogous *lpsA1* and *lpsA2* genes (7, 17). We failed to extend the *C. fusiformis* sequence information in this direction by genome walking using thermal asymmetric interlaced PCR with genomic DNA of *C. fusiformis* as the template as well as identification of any cosmid that would extend this region. Apparently, the region downstream of Cf*dmaW* is refractory to PCR or perhaps is telomere associated.

Southern blot analysis (Fig. 4), as well as PCR approaches (data not shown), revealed no *lpsA* homologues in the *C. fusiformis* genome. The Cp*lpsA1* probe (14) hybridized to at least two EcoRI fragments from the genome of *C. purpurea* P1, but there was no detectable signal from *C. fusiformis* genomic DNA. In contrast, the Cp*dmaW* probe cross-hybridized with Cf*dmaW* (as well as with a paralogue in the *C. purpurea* genome), but not with the homologues in *Neotyphodium* species or *Balansia obtecta*. This observation is in keeping with previously published phylogenetic relationships, which group the *Claviceps* species closer to each other than to other genera in the family. Therefore, the observation that the Cp*lpsA* probe hybridized detectably to the *lpsA* genes in the *Neotyphodium* species and *B. obtecta* but not to DNA from *C. fusiformis* indicates that the latter species is devoid of detectable *lpsA* homologues. It has been reported that deletion of *lpsA* in a *Neotyphodium* species eliminates production of the ergopeptine ergovaline, as well as lysergic acid amide (14). The lack of functional lysergyl peptide synthetase genes in *C. fusiformis* is not surprising because this species does not synthesize the enzyme substrate, lysergic acid, or its derivatives. In all, we identified one pseudogene (Cf*lpsB*$^{\Psi}$) and eight potentially functional genes in the *C. fusiformis EAS* cluster that were homologues of genes in the *C. purpurea EAS* cluster. These genes included *dmaW* and *cloA*, as well as genes potentially encoding a flavin mononucleotide-containing oxidoreductase (*easA*), a catalase (*easC*), a dehydrogenase (*easD*), a flavin adenine dinucleotide-containing monooxygenase (*easE*), a methyltransferase (*easF*), and an oxidoreductase/epimerase (*easG*). However, the absence of paspalic acid and lysergic acid in *C. fusiformis* raised the question of whether Cf*cloA* was functional.

Functional characterization of the *cloA* and *lpsB* homologues from *C. fusiformis*. To test their functionality, the *cloA* and *lpsB* genes of *C. fusiformis* were used in an attempt to complement the corresponding deletion mutants of *C. purpurea*. A Δ*lpsB* deletion mutant (3) was transformed with the entire Cf*lpsB*$^{\Psi}$ pseudogene, and its integration (including upstream and downstream regions) into the *C. purpurea* genome was confirmed by PCR and Southern blot analysis (data not shown). The transformed mutants were then cultivated under EA-producing conditions, and the expression of the *EAS* cluster genes was examined by Northern analysis. Cf*lpsB*$^{\Psi}$ was expressed in transformed *C. purpurea*, but the transcript was much smaller than the *C. purpurea* homologue (data not shown). This was expected because of the apparent 3′ truncation of the coding sequence, as mentioned above. Extraction of EA from the cultivation medium and HPLC analysis showed the same alkaloid spectrum in the transformants ($n = 6$) as in the deletion mutant (data not shown), confirming that the gene was nonfunctional.

The same approach was used in an attempt to complement the Δ*cloA* mutant of *C. purpurea* (8) with Cf*cloA*. Integration of Cf*cloA* in *C. purpurea* genomic DNA was also confirmed by

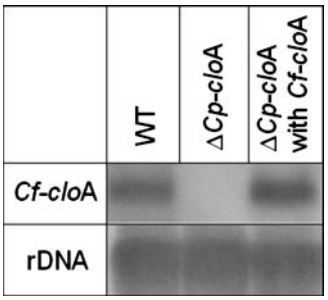

FIG. 5. Heterologous expression of Cf*cloA* in *C. purpurea* strains, including the wild type (P1) (WT), a ΔCp*cloA* mutant, and a transformant carrying the full-length Cf*cloA* gene.

PCR and Southern blot analysis (data not shown). Northern analysis showed that Cf*cloA* mRNA was expressed in the transformant (Fig. 5). However, alkaloid profiles showed no differences between the deletion mutant and the transformants (n = 10) (data not shown), indicating that Cf*cloA* does not encode a functional homologue of Cp*cloA*. These analyses show that the two *C. fusiformis* genes are expressed in the *C. purpurea* background (as they are in *C. fusiformis*); i.e., regulation of their transcription is normal, but the products that they encode seem to be inactive. This explains why the biosynthetic pathway terminates at elymoclavine in *C. fusiformis* (Fig. 1).

Modification of the alkaloid spectrum of *C. fusiformis* by a *C. purpurea* gene. If the production of more complex EA by *C. fusiformis* is limited by the lack of a functional *cloA*, then the introduction of the functional *cloA* gene from *C. purpurea* should enable *C. fusiformis* to synthesize paspalic acid and lysergic acid. The construct used for complementation of *C. fusiformis* was previously used for restoration of the wild-type profile of the Δ*cloA* mutant of *C. purpurea* (8). Integration of Cp*cloA* into the *C. fusiformis* genome was confirmed by PCR and Southern blot analysis, and expression of the gene was verified by RT-PCR (data not shown). Five of the transformants containing the *C. purpurea* gene were analyzed under EA-inducing conditions. Since the expected intermediates (paspalic acid and lysergic acid) are normally not secreted, mycelial extracts were also analyzed. As shown in Fig. 6, mycelial extracts of transformants indeed contained paspalic acid and lysergic acid, which were completely undetectable in the untransformed *C. fusiformis* recipient strain. The identity of the TLC peaks was confirmed by liquid chromatography-mass spectrometry analyses (Fig. 7). These results demonstrated that CloA converts elymoclavine to paspalic acid and confirmed that this step is missing in the *C. fusiformis* wild type.

The relatively small amount of lysergic acid present in the extracts could result from end product inhibition, as has been postulated to explain the alkaloid spectrum of the Cp*cloA* mutant (8). As in this mutant, in *C. fusiformis* the end product of alkaloid biosynthesis, elymoclavine, is present at low levels compared to the levels of its immediate precursor, agroclavine, probably due to feedback inhibition of the agroclavine-oxidizing enzyme. In the complemented strain, elymoclavine accumulated to higher levels than lysergic acid, conceivably due to feedback inhibition by lysergic acid (Fig. 8).

Evolution of *EAS* clusters in *Claviceps* species. Here we show that *C. fusiformis* has homologues of nine of the *C. purpurea* *EAS* genes previously identified but that two of these genes (Cf*cloA* and Cf*lpsB*$^{\Psi}$) apparently encode nonfunctional products. It seems likely that the other seven Cf*EAS* genes are functional; they are most likely involved in clavine biosynthesis, since homologues of *easA*, *easC*, *easD*, *easE*, *easF*, *easG*, and *dmaW* are also present in the fumigaclavine biosynthesis gene cluster of *Aspergillus fumigatus* (4, 11). The EA profile of *A. fumigatus* (order Eurotiales) includes festuclavine and fumigaclavines but no lysergic acid or lysergic acid amides. Furthermore, *A. fumigatus* lacks identifiable homologues of *cloA*, *lpsA*, *lpsB*, or *lpsC*, suggesting that an ancestral gene cluster acquired *cloA* and the NRPS genes on the evolutionary lineage to the plant-associated Clavicipitaceae. In contrast, the presence of an *lpsB* pseudogene in *C. fusiformis* suggests that the common ancestor of *C. purpurea* and *C. fusiformis* synthesized amides of lysergic acid and that the trait was lost in the *C. fusiformis* lineage. An *lpsA* homologue has also been found in *Epichloë* (*Neotyphodium*) species, where it is required for formation of ergovaline (14). Furthermore, deletion of an *lpsB* homologue in *Epichloë festucae* led to accumulation of lysergic acid, as well as accumulation of the clavine compound 6,7-secolysergine, whereas the end product ergovaline was completely missing, as were lysergyl alanine and ergine, both of which are derivatives of lysergic acid (5). Interestingly, Fleetwood et al. also identified several long terminal repeat retrotransposons and nonautonomous transposable elements in the intergenic regions of the alkaloid cluster of *Neotyphodium lolii*, which could be the reason for rearrangement events in the overall structure of the alkaloid cluster (5). Also, *Balansia* species and clavicipitaceous symbionts of *Ipomoea* species

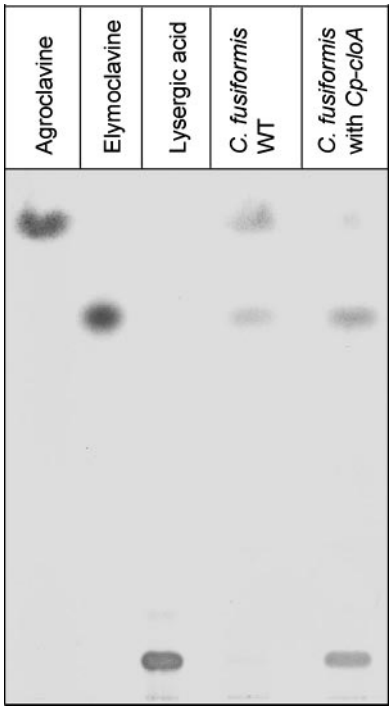

FIG. 6. TLC analyses of ergot alkaloids from the *C. fusiformis* wild type (WT) and a transformant carrying the Cp*cloA* gene. Culture extracts were chromatographed on TLC plates, visualized by using van Urk's reagent, and identified by comparison with pure standards.

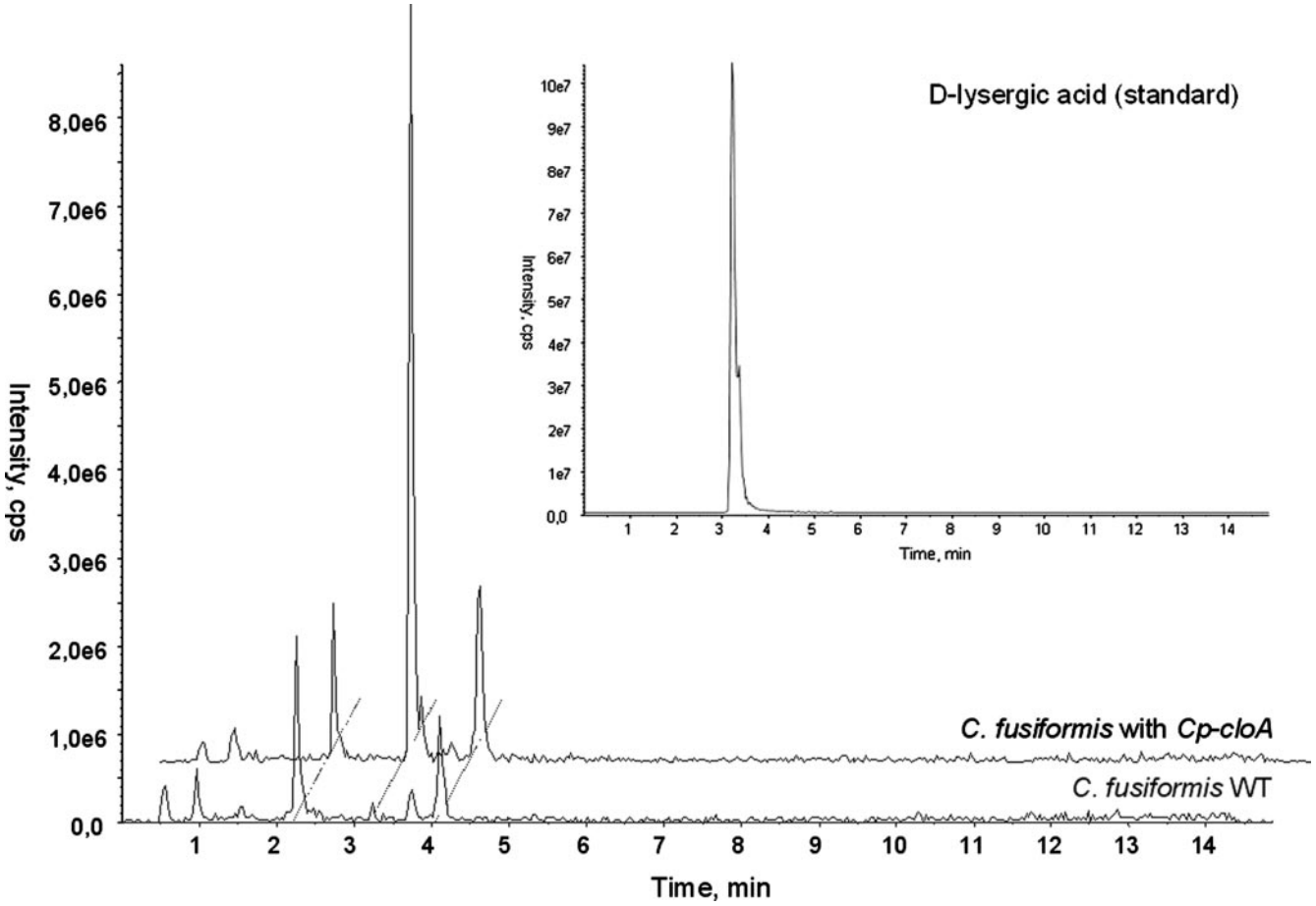

FIG. 7. Liquid chromatography-mass spectroscopy analyses of ergot alkaloids from the *C. fusiformis* wild type (WT) and a transformant carrying the Cp*cloA* gene. The inset shows the migration of the lysergic acid standard.

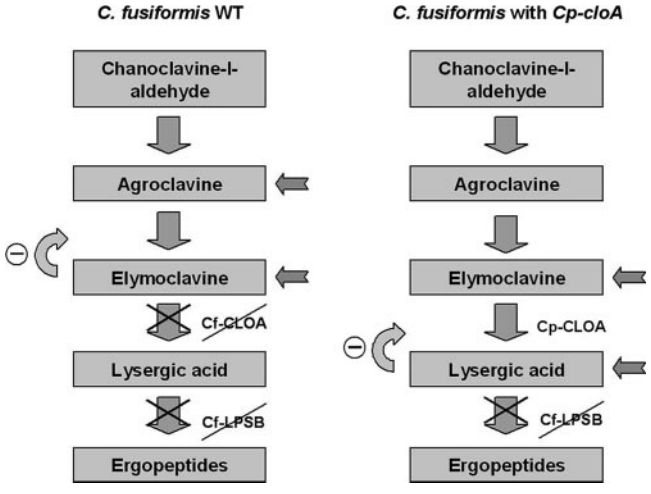

FIG. 8. Diagram of ergot alkaloid biosynthesis in the *C. fusiformis* wild type (WT) and a transformant carrying the Cp*cloA* gene. In the *C. fusiformis* wild type the pathway ends at elymoclavine due to a nonfunctional Cf*cloA* gene. In addition to elymoclavine, agroclavine accumulates, perhaps due to feedback inhibition. In the *C. fusiformis* transformant carrying a functional Cp*cloA* gene, lysergic acid and elymoclavine accumulate. The absence of functional *lpsA* and *lpsB* genes in *C. fusiformis* apparently precludes the synthesis of ergopeptines in this transformant.

(plant family Convolvulaceae) produce the ergopeptine ergobalansine, which, like ergotamine and ergovaline, comprise lysergic acid and three L-amino acids in a cyclol-lactone ring system (9, 15, 19). Therefore, it appears that the common ancestor of most or all plant-associated Clavicipitaceae species had one or more *lpsA* homologues, which were lost in the *C. fusiformis* lineage.

It is interesting to speculate about the order in which functions of *cloA* and *lpsB* were lost in the *C. fusiformis* lineage. The sequence divergence of the *lpsB* genes in the two species is slightly greater than that of the *cloA* genes. This may be because of an earlier loss of *lpsB* function or, alternatively, because functional *lpsB* is less conserved. If we speculate that *lpsB* function was lost first, a reasonable scenario would start with the rearrangement of the *EAS* cluster segment containing it, with concomitant deletion of most of the third domain. This might have provided selection for the frameshift mutations that eliminate the potential for production of anything other than a small peptide product from this gene. Furthermore, the loss of functional *lpsB* could have led to accumulation of lysergic acid as the end product. Interestingly, typical EA-producing Clavicipitaceae species tend to produce either lysergyl amides and ergopeptines or clavines, but they accumulate little lysergic acid. Perhaps the latter compound is detrimental to

the producing fungi, or perhaps it is simply less beneficial as a protectant than either the clavines or lysergyl amides and ergopeptines. In either case, loss of *lpsB* would lead to selection for loss of functional *cloA*.

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