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RESEARCH ARTICLE

Ergot cluster-encoded catalase is required for synthesis of chanoclavine-I in *Aspergillus fumigatus*

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Abstract Genes required for ergot alkaloid biosynthesis are clustered in the genomes of several fungi. Several conserved ergot cluster genes have been hypothesized, and in some cases demonstrated, to encode early steps of the pathway shared among fungi that ultimately make different ergot alkaloid end products. The deduced amino acid sequence of one of these conserved genes (*easC*) indicates a catalase as the product, but a role for a catalase in the ergot alkaloid pathway has not been established. We disrupted *easC* of *Aspergillus fumigatus* by homologous recombination with a truncated copy of that gene. The resulting mutant (Δ*easC*) failed to produce the ergot alkaloids typically observed in *A. fumigatus*, including chanoclavine-I, festuclavine, and fumigaclavines B, A, and C. The Δ*easC* mutant instead accumulated *N*-methyl-4-dimethylallyltryptophan (*N*-Me-DMAT), an intermediate recently shown to accumulate in *Claviceps purpurea* strains mutated at *ccsA* (called *easE* in *A. fumigatus*) (Lorenz et al. Appl Environ Microbiol 76:1822–1830, 2010). A Δ*easE* disruption mutant of *A. fumigatus* also failed to accumulate chanoclavine-I and downstream ergot alkaloids and, instead, accumulated *N*-Me-DMAT. Feeding chanoclavine-I to the Δ*easC* mutant restored ergot alkaloid production. Complementation of either Δ*easC* or Δ*easE* mutants with the respective wild-type allele also restored ergot alkaloid production. The *easC* gene was expressed in *Escherichia coli*, and the protein product displayed in vitro catalase activity with H_2O_2 but did not act, in isolation, on *N*-Me-DMAT as substrate. The data indicate that the products of both *easC* (catalase) and *easE* (FAD-dependent oxidoreductase) are required for conversion of *N*-Me-DMAT to chanoclavine-I.

Keywords Ergot alkaloids · Mycotoxin · Clavines · Catalase · Gene cluster · *Aspergillus fumigatus*

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Introduction

Several fungi produce ergot alkaloids that are important in agriculture and medicine (Schardl et al. 2006; Lorenz et al. 2009; Panaccione 2010). Among these fungi are plant-associated pathogens and symbionts from the family Clavicipitaceae (order Hypocreales), which typically produce lysergic acid derivatives, and the opportunistic human pathogen *A. fumigatus* (family Trichocomaceae, order Eurotiales), which produces a family of simpler, clavine-type ergot alkaloids including chanoclavine-I, festuclavine, fumigaclavine B, fumigaclavine A, and fumigaclavine C. A thorough understanding of the genes and enzymes involved in ergot alkaloid biosynthesis may lead to innovations in agriculture and medicine.

Ergot alkaloids are derived via prenylation of tryptophan, with subsequent N-methyltransferase and redox reactions resulting in tricyclic and tetracyclic ergot alkaloids (Schardl et al. 2006; Lorenz et al. 2009; Panaccione 2010)

(Fig. 1). The critical intermediate chanoclavine-I is the first tricyclic ergot alkaloid formed and is typically the earliest pathway intermediate detectable in ergot alkaloid-producing fungi. The immediate oxidation product of chanoclavine-I, chanoclavine-I-aldehyde, is the final intermediate that is shared in the biosynthesis of the varied ergot alkaloid profiles observed among different fungi (Coyle et al. 2010; Cheng et al. 2010a). Thus, ergot alkaloid-producing fungi share several early steps before their ergot alkaloid pathways diverge to yield different end products.

Several genes in the ergot pathway have been cloned and functionally analyzed. The ergot pathway genes are found in clusters in the genomes of the producing fungi (Tudzynski et al. 1999; Coyle and Panaccione 2005; Unsöld and Li 2005; Lorenz et al. 2007; Fleetwood et al. 2007). Those genes that are common to clusters among producers have been hypothesized, and in some cases demonstrated, to encode the early, shared steps in the pathway. Evidence supporting this hypothesis includes functional analysis by gene knockout or heterologous expression of *dmaW* (Wang et al. 2004; Coyle and Panaccione 2005; Unsöld and Li 2005), *easF* (Rigbers and Li 2008), *ccsA* (synonym *easE*) (Lorenz et al. 2010), *easD* (Wallwey et al. 2010a), *easA* (Coyle et al. 2010; Cheng et al. 2010a, b), and FgaFS (synonym *easG*) (Wallwey et al. 2010b; Cheng et al. 2010a). Additional genes found in the ergot gene clusters are hypothesized to also contribute to the pathway. One such additional gene is shared among all known ergot clusters and encodes a product with structural similarity to peroxisomal catalases (Haarmann et al. 2005; Coyle and Panaccione 2005; Unsöld and Li 2005). In this paper, we report the functional analysis of this open reading frame by gene disruption and heterologous expression.

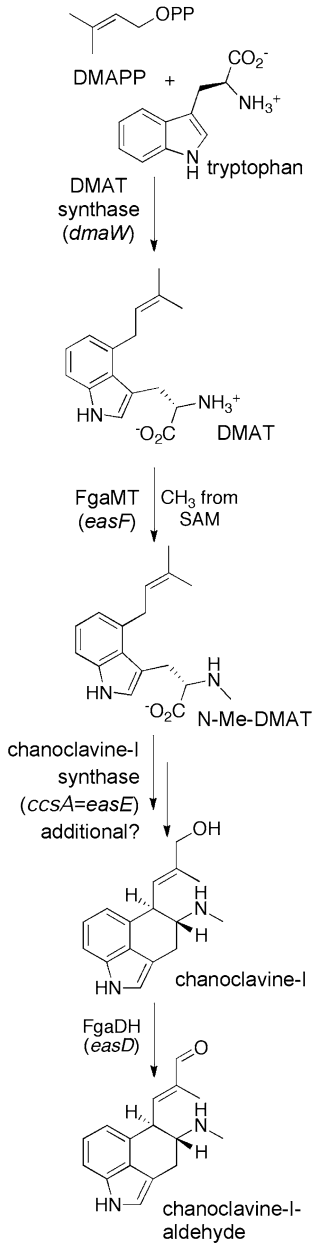

Fig. 1 Early steps of the ergot alkaloid pathway that are shared among diverse ergot alkaloid producers. Enzyme and gene names are provided to the left of arrows indicating catalysis (Wang et al. 2004; Coyle and Panaccione 2005; Unsöld and Li 2005; Schardl et al. 2006; Rigbers and Li 2008; Lorenz et al. 2010; Wallwey et al. 2010a). *DMAPP* dimethylallylpyrophosphate, *Trp* tryptophan, *DMAT* dimethylallyltryptophan, *SAM* S-adenosylmethionine

Materials and methods

Gene disruption and complementation

Aspergillus fumigatus genomic DNA was isolated according to the Gene Clean Spin Protocol (Bio 101, Vista, CA). A 655-bp internal fragment of the *easC* catalase gene (GenBank accession XM_751047) representing bases 338–992 of the 1,560-bp of the coding sequences was PCR amplified from primers easC-F (5′-GGCTGGAGCGAGGTTCTG-3′) and easC-R (5′-TCGGCGAAGAAGTTATCCG-3′). The 50-μL reaction contained 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 200 μM of each deoxyribonucleotide triphosphate, 1 μM of each primer, and 0.5 units of Taq DNA polymerase (Promega, Madison, WI). The reaction conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final incubation of 72°C for 5 min. The resulting product was ligated into the TA cloning vector PCR 2.1 (Invitrogen, Carlsbad, CA) to make a 4.6-kb catalase gene disruption construct (pEASC1; Fig. 2a). Prior to transformation into *A. fumigatus*, pEASC1 was linearized with *Sac*II, which cuts once within the catalase gene coding sequence. This linearized DNA was transformed into *A. fumigatus* protoplasts as described by Coyle

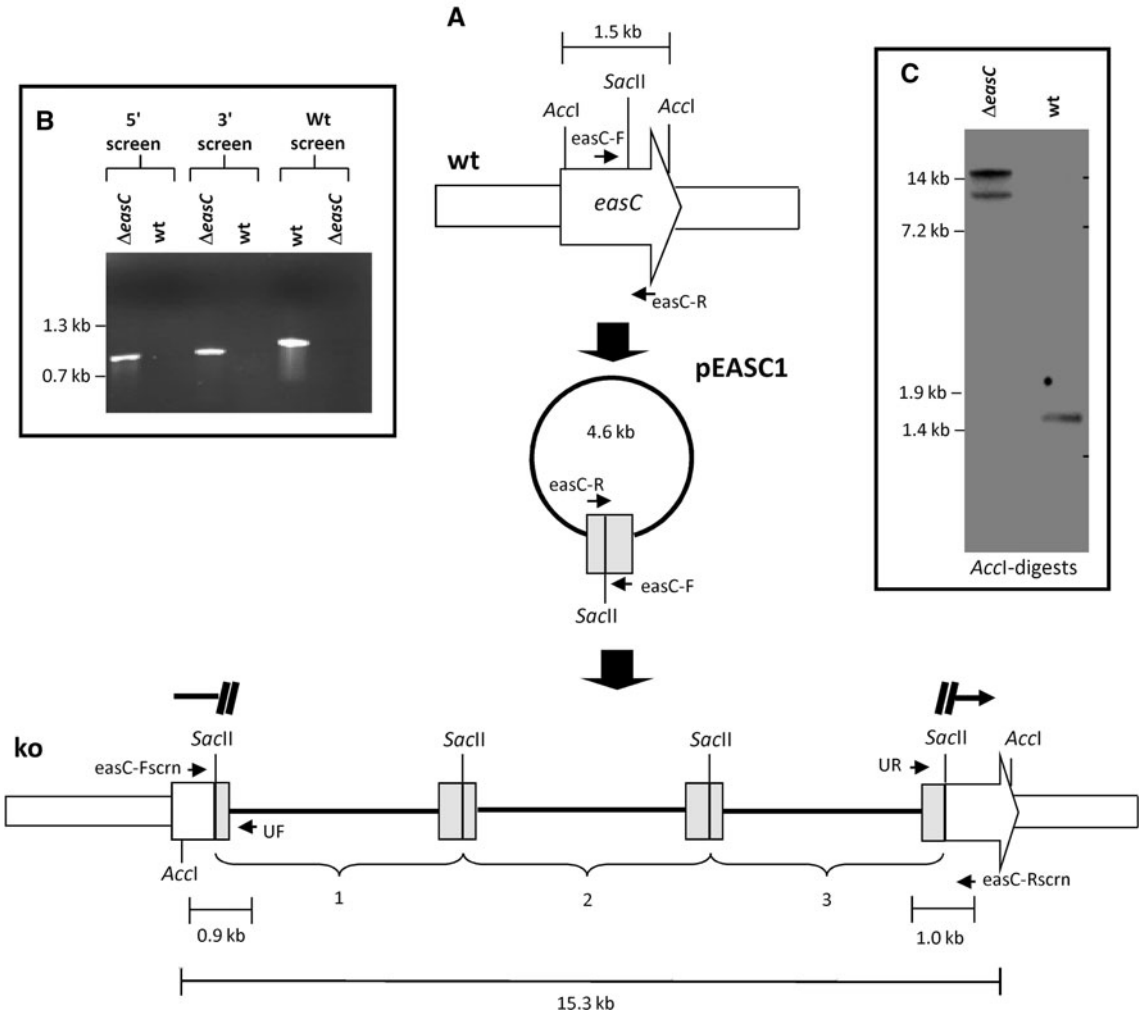

Fig. 2 Disruption strategy for *easC*. **a** EasC-F and easC-R primed amplification of an internal region of *easC*. Gene disruption construct pEASC1 contains this internal fragment and was linearized at a unique *Sac*II site prior to transformation. (The *Sac*II site indicated is unique to the PCR fragment that was ligated into the disruption construct. An additional *Sac*II site occurs toward the 3′ end of the gene, outside of the region that was PCR amplified; the second *Sac*II site is not a factor in any of the analyses and is not labeled in the figure.) The bottom panel illustrates insertion of three copies of the pEASC1 within the native copy of *easC* and the positions of primers used in PCR experiments to demonstrate integration at this locus. **b** Homologous recombination of transforming DNA at *easC* was demonstrated by PCR with primers easC-Fscrn and UF confirming integration at the 5′-end of the gene and primers UR and easC-Rscrn confirming integration at the 3′-end. Primer locations are shown in the bottom portion of part A. A screen for the presence of the wild-type allele was primed from easC-Fscrn and easC-Rscrn; amplification did not occur from the disrupted allele due to the increase in template length. PCR of non-transformed (wild-type) DNA gives a product of 1,036 bp. **c** Integration of transforming DNA at the targeted site was confirmed by Southern blot hybridization. Fungal DNA extracts were digested with *Acc*I and hybridized with a digoxigenin-dUTP labeled *easC* probe (corresponding to *gray-shaded* sequences). The relative mobilities of relevant fragments of *Bst*EII-digested bacteriophage lambda DNA are indicated on the left in panels B and C

et al. (2010). Transformants were purified to nuclear homogeneity by starting cultures from single uninucleate conidia.

Transformants were screened for integration of the disruption construct at the native *easC* locus by two types of PCR assays. One assay was designed to amplify across the 5′-junction or 3′-junction of an allele resulting from homologous recombination of the disruption construct at the native copy of *easC*. Template DNA was amplified with primers easC-Fscrn (5′-GAATTCGAGGTATTGATCTCC-3′) or easC-Rscrn (5′-AGCCAGGCAAAGATCCATGT T-3′), paired with pCR2.1-derived primers UF (5′-TG TAAAACGACGGCCAGTGAAT-3′) and UR (5′-AGCT ATGACCATGATTACGCCA-3′), respectively (Fig. 2a). The PCR was as described above for preparation of the gene disruption construct. In the second type of PCR assay, template DNA was primed with oligonucleotides easC-Fscrn and easC-Rscrn (sequences provided above), which prime amplification of a 1,036-bp product from the wild-type allele. In strains in which the knockout construct has integrated by homologous recombination, the primer

annealing sites are too far apart and no PCR product can be observed.

A similar strategy was used to disrupt *easE* of *A. fumigatus* (GenBank accession XM_751049) (Fig. 3). The gene labeled *easE* in the *A. fumigatus* ergot gene cluster (Schardl et al. 2006; Panaccione 2010) is homologous to the gene named *ccsA* in *C. purpurea* (Lorenz et al. 2010). An internal (incomplete) fragment of *easE* coding sequences was amplified by PCR primed with the oligonucleotides easE-F (5′-CCAGATACATTGCCATCGCATG-3′) and easE-R (5′-TGTTCCAACTGCTTGGCCAGAT-3′) and with reaction conditions described above for amplification of *easC*, but with an annealing temperature of 57°C. A disruption construct was generated by ligating the PCR amplicon into the T/A overlap vector pCR2.1 (Invitrogen, Carlsbad, California). The 4.8-kb construct (pEASE1) was linearized in the *easE* sequences with *Bsp*EI and transformed into *A. fumigatus* as described above. Transformants were screened by the PCR strategies described above, but with the following primers: the 5′ flank of the integration site was screened with primers UF (5′-TGTAAAACGACGGC CAGTGAAT-3′) and easE-Fscrn (5′-ACTGAAGACGAG ACATGTCATG -3′), and the 3′ flank of the integration site was screened with primers UR (5′-AGCTATGACCAT GATTACGCCA-3′) and easE-Rscrn (5′-TTCCTGTACG ACACTCTGAAGT -3′). The presence of a wild-type allele was tested with primers easE-Fscrn and easE-Rscrn (both described above).

Integration of the transformation constructs also was analyzed by Southern hybridization as described previously (Coyle and Panaccione 2005), except for the substitution of gene-specific probes. Each gene-specific probe was made by amplifying wild-type *A. fumigatus* genomic DNA via PCR from primer pairs easC-F and easC-R for *easC*, and easE-F and easE-R for *easE*, according to the PCR conditions described above for those primer combinations, but substituting 1 × DIG DNA LabelingMix (Roche, Mannheim, Germany) for unlabeled dNTPs. Transformant and wild-type genomic DNA samples were digested with *Acc*I for both *easC* and *easE*.

The Δ*easC* disruption strain was complemented with the wild-type allele of *easC* amplified from primers WeasC-F (5′-CTAATATTGCAGGACTCCGAT-3′) and WeasC-R (5′-GGTACATCAAATCTTGCTGGA-3′). The 3,078-bp product contained the entire coding sequences of *easC* along with 934 bp of 5′-flanking sequences and 524 bp of 3′-flanking sequences. Similarly, the Δ*easE* mutant was complemented with a 4,594-bp fragment amplified from primers WeasE-F (5′-CTGCTCCAGCAGGTAGATCTT GATTC-3′) and WeasE-R (5′-TAGAGGAGCACCCAGC TGAATGTCCT-3′). This fragment contained the *easE* coding sequences along with 1,445 bp of 5′-flanking sequences and 1,177 bp of 3′-flanking sequences. Each PCR product was cotransformed into their respective gene-disrupted mutant along with a phleomycin resistance plasmid, pBCphleo (Fungal Genetics Stock Center, University of Missouri-Kansas City, Kansas City, MO), as described previously (Coyle et al. 2010). To test for the presence of the original disruption construct, PCR reactions were performed as described above (e.g., easE-F and UF). An additional PCR screen was performed on each complemented transformant to test for the presence of the introduced wild-type allele. These reactions were primed with primer pairs and the reaction conditions stated above (i.e., primers WeasE-F and WeasE-R, or WeasC-F and WeasC-R).

Ergot alkaloid analysis

Sporulating cultures of *A. fumigatus* grown on potato dextrose agar were extracted with 80% methanol and analyzed by HPLC with fluorescence detection as described by Panaccione and Coyle (2005). Identities of ergot alkaloids in wild-type *A. fumigatus* (chanoclavine-I, festuclavine, and fumigaclavines A, B, and C) have been established previously (Panaccione and Coyle 2005; Coyle and Panaccione 2005; Coyle et al. 2007, 2010). *N*-methyl-4-dimethylallyltryptophan (*N*-Me-DMAT) was identified by ESI LC–MS (Coyle et al. 2010) and by co-elution with the standard prepared as described below.

Feeding experiments with exogenously supplied chanoclavine-I

Cultures of a catalase gene disruptant (Δ*easC*) were fed exogenous chanoclavine-I to test whether the mutant's pathway was functional at steps downstream from chanoclavine-I. A *dmaW* gene disruptant strain (Δ*dmaW*), which is deficient in the first step of the pathway (Coyle and Panaccione 2005), served as a positive control. Triplicate cultures were started from 60,000 conidia in 200 µL of lactose-malt extract-arginine broth (Spilsbury and Wlikinson 1961) in 2-mL microcentrifuge tubes. Chanoclavine-I (26.7 nmol in 1 µL of methanol) was added to the treatment tubes, and 1 µL of 100% methanol was added to control cultures. All cultures were incubated at room temperature for 3 days. Alkaloids were extracted from each tube by adding 800 µL of methanol and ten 3-mm diameter glass beads and agitating the cultures in a Fast Prep FP120 (Q-biogene, Irving, CA) at 6 m/s for 30 s. Samples were filtered through 0.2 µM nylon filters and analyzed by HPLC as described above.

Preparation of *N*-methyl-4-dimethylallyltryptophan (*N*-Me-DMAT)

N-Me-DMAT was prepared by incubating *N*-methyl-L-tryptophan (L-abrine) with heterologously expressed

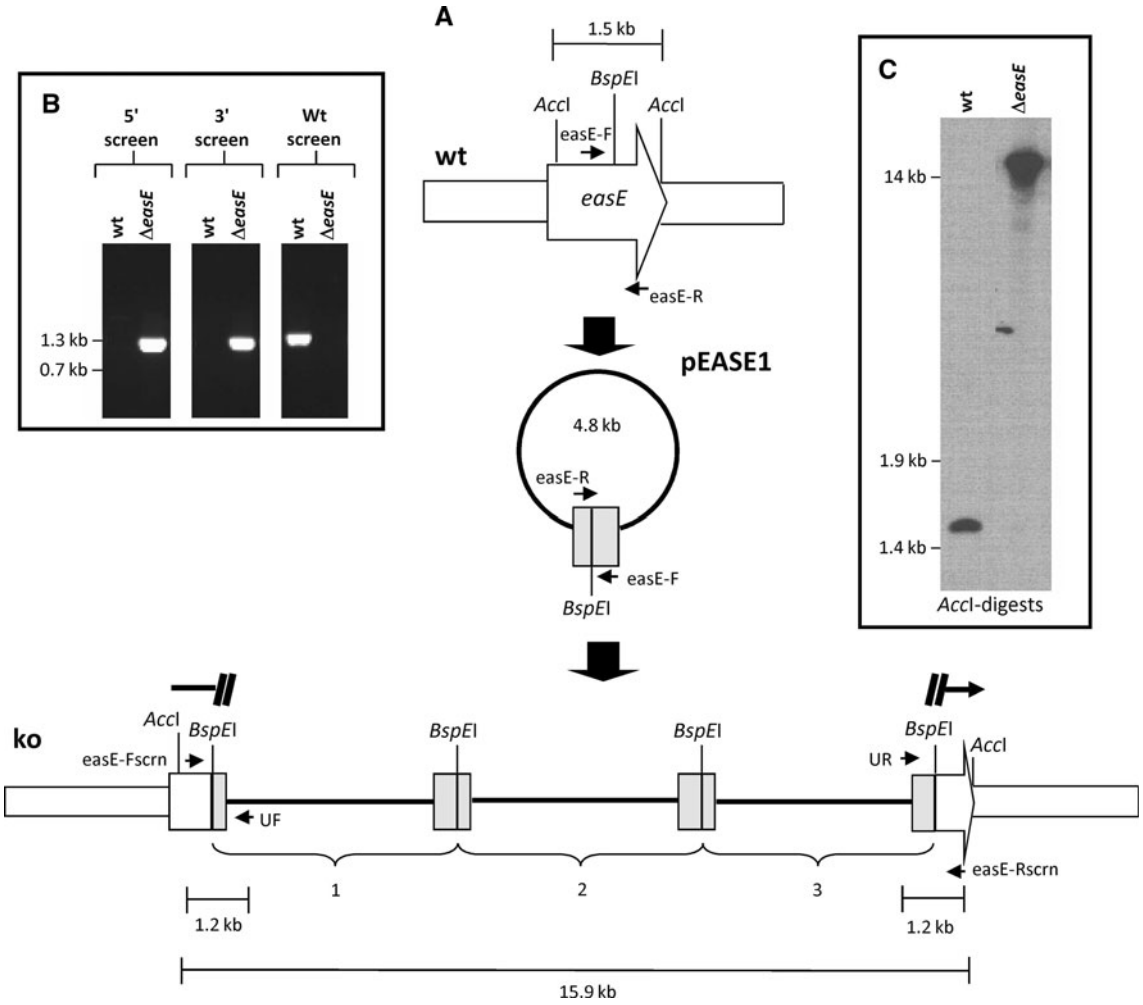

Fig. 3 Disruption strategy for *easE*. **a** EasE-F and easE-R primed amplification of internal region of *easE*. Gene disruption construct pEASE1 contains this internal fragment and was linearized at a unique *BspE*I site prior to transformation. The bottom panel illustrates the insertion of three copies of the pEASE1 within genomic *easE* and the positions of primers used in PCR experiments to demonstrate integration at this locus**. b** PCR with primers easE-Fscrn and UF spans the 5′-end of the disrupted allele, and PCR from primers UR and easE-Rscrn spans the 3′-end of the disrupted allele, confirming integration of disruption constructs at the native *easE* locus. PCR primed from easE-Fscrn and easE-Rscrn demonstrates the presence of an intact allele in the wild type, but not in the Δ*easE* mutant, due to the increase in template length in the disrupted allele. Primer locations are shown in the bottom portion of part A. **c** Integration of disruption constructs at *easE* is illustrated by Southern blot hybridization. Fungal genomic DNA extracts was digested with *Acc*I and hybridized with a digoxigenin-dUTP labeled *easE* probe (corresponding to *gray-shaded* sequences). Only the *Acc*I sites relevant to the Southern blot are shown; additional *Acc*I sites flanking the indicated sites (toward the margins of the figure) are omitted because the probe does not hybridize to those regions. The relative mobilities of relevant fragments of *Bst*EII-digested bacteriophage lambda DNA are indicated on the left in panels B and C

DMAT synthase from *C. purpurea*. DMAT synthase coding sequences were amplified from the *C. purpurea* gene, which had been previously cloned into pKAES154 (Wang et al. 2004) in a PCR reaction primed from oligonucleotides 5′-TT*CATATG*GGTGTGTACGAAATTTTGAGTCTGA-3′ (with *Nde*I restriction site italicized) and 5′-GA*CTCGA* GTTAAAGCTTCTTCGTTGAGAGTTCACAGCGCCG G-3′ (*Xho*I restriction site italicized). The amplified gene was cloned into the *Nde*I/*Xho*I site of pET-28a(+) expression vector (EMD Biosciences, Merck, Darmstadt, Germany) as an N-His_6 construct. Expression was carried out in *Escherichia coli* strain BL-21(DE3) grown in LB medium supplemented with kanamycin (50 μg/mL). Optimal conditions for N-His_6-tagged DMAT synthase overexpression were by induction with 5 μM IPTG at 15°C for 68 h. The enzyme was purified by Ni–NTA affinity chromatography, where the purest fraction was obtained in elution buffer (100 mM imidazole, 20 mM Tris–HCl, 300 mM NaCl, and 10% (v:v) glycerol, pH 8.0). This fraction was collected and buffer exchanged by dialysis into 50 mM Tris–HCl, 100 mM NaCl, 1 mM dithiothreitol (DTT), and 10% (v:v) glycerol, pH 8.0.

DMAT synthase was assayed by following previously reported conditions (Gebler et al. 1992; Unsöld and Li

2005), and substrate turnover and product formation were analyzed by HPLC and ESI LC–MS. DMAT synthase also converted N-Me-tryptophan (L-abrine) to a compound with a mass of 287.17, which is consistent with the prenylated product, *N*-Me-DMAT.

In vitro expression and activity of EasC

Total RNA was extracted by the Trizol procedure (Invitrogen, Carlsbad, CA), and cDNA was prepared with Creator SMART MMLV reverse transcriptase (Clontech, Mountain View, CA). A pair of oligonucleotide primers was used to prime amplification of the *easC* gene: forward primer 5′-TTCCATGGGC*CATATG*CTAATTGAGCGTGGGTTATTGTC-3′ (with *Nde*I restriction site italicized) and reverse primer 5′-TT*CTCGAG*TTAAGCTTTGAGCCTGGAAAGAGAGACTTGTTTG-3′ (*Xho*I restriction site italicized). The amplified *easC* gene was inserted as an *Nde*I/*Xho*I fragment into pET-28a(+) (Novagen, Merck, Darmstadt, Germany) to prepare an N-His_6 construct. Expression was carried out in LB medium supplemented with kanamycin (50 μg/mL) and with the addition of precursors for heme formation at final concentrations of 0.4 mM aminolevulinic acid and 0.2 mM ferric ammonium sulfate. *Escherichia coli* BL-21(DE3) cells were grown to an OD_{600} of 0.8 prior to induction with IPTG (5 μM) and grown for 68 h at 15°C prior to harvesting. EasC was purified by chromatography on Ni–NTA agarose (Qiagen, Valencia, CA). Fractions containing pure EasC, as demonstrated by SDS–PAGE, were collected and exchanged with dialysis buffer 50 mM Tris–HCl, 100 mM NaCl, 1 mM dithiothreitol (DTT) and 10% (v:v) glycerol, pH 8.0. The catalase assay was conducted as described by Aebi (1984) in a quartz cuvette with 0.1 μM EasC and 10 mM H_2O_2 in dialysis buffer. Expressed and purified EasC (0.2 μM) was incubated with *N*-Me-DMAT for 2 h (50 mM Tris–HCl, pH 7.5, 5 mM $CaCl_2$), with and without the addition of 10 mM H_2O_2. Potential disappearance of starting material and formation of any new products were monitored by HPLC and ESI LC–MS.

Results

A role for *easC* in the ergot alkaloid pathway

Functional analysis of *easC* was conducted by gene disruption. Since the disruption construct contained an internal fragment of the coding sequences of *easC*, integration of that fragment by homologous recombination would truncate the native copy of *easC* at both the 5′ and 3′ ends (Fig. 2a). Integration of the disruption construct at *easC* was demonstrated by PCR analysis in one of 20 transformants analyzed. PCR amplification primed from easC-Fscrn, which anneals to *easC* sequences 5′ of the desired integration site, and UF, which anneals to pCR2.1 vector sequences, produced a product of 923 bp from 1 transformant (Δ*easC*) among the 20 investigated transformants (Fig. 2b). A PCR product of this length would only be amplified from a reaction primed with this primer pair if the disruption construct had integrated at *easC*. Similarly, a PCR reaction designed to span the 3′ junction of the allele predicted to result from homologous recombination was primed from oligonucleotides UR and easC-Rscrn. PCR with this primer pair resulted in a product of 962 bp, as expected only if the disruption construct had integrated at *easC* by homologous recombination (Fig. 2b). PCR analysis with primers easC-Fscrn and easC-Rscrn, which prime amplification of a 1,036-bp fragment from wild type, produced no product from the Δ*easC* disruptant (Fig. 2b), indicating a lack of a wild-type allele in this transformant. The presence of multiple gene disruption constructs at *easC* and possibly elsewhere in the genome was indicated by Southern hybridization of genomic DNA digested with *Acc*I, which does not cut within the *easC* sequences used as a probe. DNA sequence analysis revealed that the coding sequences used to direct the disruption of *easC* were contained on a 1,456-bp *Acc*I fragment in the wild type. A fragment of this length was evident in the Southern blot of wild-type DNA, but a similar fragment was not detectable in the Δ*easC* disruption strain (Fig. 2c). Instead, *easC* sequences were found on fragments of approximately 10 kb and 15 kb in the Δ*easC* mutant (Fig. 2c), indicating integration of two or three copies of the 4.6-kb disruption construct into the 1,456-bp fragment of *easC* in that strain (Fig. 2a). One of the fragments also may have resulted from ectopic integration of another copy of the gene disruption construct.

Methanolic extracts of conidia of the Δ*easC* disruption strain were compared with extracts from conidia of wild-type *A. fumigatus* by HPLC. The ergot alkaloids chanoclavine-I, festuclavine, fumigaclavine B, fumigaclavine A, and fumigaclavine C were not detected in extracts from the Δ*easC* disruption strain (Fig. 4). These ergot alkaloids were detected in extracts of the wild-type strain that had been grown on the same medium and environmental conditions as the Δ*easC* strain. In addition, the Δ*easC* mutant extracts contained a peak at 46 min that was not present in wild-type extracts. This compound was analyzed by mass spectrometry and shown to have a *m/z* of 287.17. Among intermediates in the ergot alkaloid pathway, this mass is consistent with the $[M + H]^+$ for *N*-Me-DMAT. *N*-Me-DMAT was recently demonstrated to accumulate in mutants of *C. purpurea* in which the gene *ccsA* (also referred to as *easE* in the ergot alkaloid gene cluster; Schardl et al. 2006) was mutated (Lorenz et al. 2010). To determine

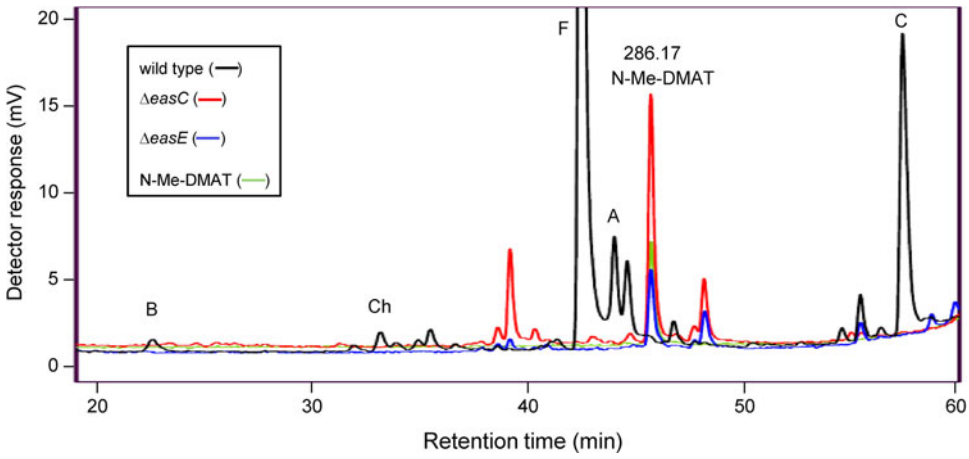

Fig. 4 HPLC analyses of wild-type *A. fumigatus* (wt; *black line*), *easC* disrupted strain (Δ*easC*; *red line*), *easE* disrupted strain (Δ*easE*; *blue line*), and prepared *N*-Me-DMAT standard (*green line*). *B* fumigaclavine B, *Ch* chanoclavine-I, *F* festuclavine, *A* fumigaclavine A, *C* fumigaclavine C. Detection was by fluorescence with excitation at 272 nm and emission at 372 nm (color figure online)

Table 1 Conversion of exogenous chanoclavine-I (26.7 nmol) into downstream ergot alkaloids by cultures of *Aspergillus fumigatus*

Culture	Ergot alkaloid (nmol; mean ± standard error)				
	Chanoclavine-I	Festuclavine	Fumigaclavine B	Fumigaclavine A	Fumigaclavine C
Δ*easC* control[a]	<d.l.[b]	<d.l.	<d.l.	<d.l.	<d.l.
Δ*dmaW* control	<d.l.	<d.l.	<d.l.	<d.l.	<d.l.
Δ*easC* + chanoclavine-I	16.0 ± 2.7	0.6 ± 0.08	0.7 ± 0.03	1.4 ± 0.03	0.03 ± 0.01
Δ*dmaW* + chanoclavine-I	19.4 ± 1.1	0.3 ± 0.01	0.6 ± 0.04	1.4 ± 0.09	0.04 ± 0.01
Medium + chanoclavine-I[c]	26.1 ± 3.7	<d.l.	<d.l.	<d.l.	<d.l.

[a] Control, cultures with no added chanoclavine-I

[b] <d.l., below detection limit

[c] Uninoculated medium with added chanoclavine-I

whether this phenotype was observed in *A. fumigatus*, we mutated *easE* of *A. fumigatus* by a strategy analogous to that described above for *easC*. PCR and Southern analyses analogous to those described for the *easC* mutant demonstrated successful integration of the *easE* disruption construct at the native *easE* locus (Fig. 3). As observed with the Δ*easC* strain, the Δ*easE* mutant lacked the typical *A. fumigatus* ergot alkaloids chanoclavine-I, festuclavine, and fumigaclavines B, A, and C (Fig. 4). Moreover the Δ*easE* mutant also accumulated an analyte with a retention time of 46 min (Fig. 4) and an *m*/*z* of 287.17 that was indistinguishable from the compound observed in the Δ*easC* disruptant. To verify the identity of this compound as *N*-Me-DMAT, an *N*-Me-DMAT standard was prepared. The prepared standard had an *m*/*z* of 287.17 and co-eluted with the analytes of the Δ*easC* and Δ*easE* disruption mutants (Fig. 4).

Strains carrying disruptions at *easC* or *easE* were complemented by transformation with wild-type alleles of the respective genes. Mutant strains transformed with the appropriate wild-type allele produced a profile of ergot alkaloids consistent with the wild-type strain of *A. fumigatus* (data not shown).

Conversion of chanoclavine-I to downstream ergot alkaloids by the Δ*easC* mutant

The ability of the Δ*easC* mutant to produce alkaloids downstream of chanoclavine-I was tested by feeding the chanoclavine-I precursor to these cultures. Cultures of the Δ*easC* disruptant and of a Δ*dmaW* mutant (Coyle and Panaccione 2005), included as a positive control strain blocked early in the ergot alkaloid pathway, converted exogenously fed chanoclavine-I into downstream ergot alkaloids typical of *A. fumigatus*. HPLC analysis of the cultures demonstrated the presence of festuclavine, and fumigaclavine B, A, and C (Table 1). Precursor feeding experiments in both Δ*easC* and Δ*dmaW* mutants resulted in downstream ergot alkaloid production with similar efficiency and in similar proportions. Incubated control medium that had been supplemented with chanoclavine-I but not inoculated with an *A. fumigatus* strain contained chanoclavine-I, but no other ergot alkaloids. Similarly, cultures of the Δ*easC* and Δ*dmaW* mutants that had not been supplemented with chanoclavine-I did not contain chanoclavine-I or downstream alkaloids.

In vitro catalase activity of heterologously expressed EasC

To test whether EasC, the protein product of *easC*, could truly function as a catalase, as its amino acid sequence predicted, we expressed the coding sequences of *easC* in *E. coli*. The expressed protein product had an estimated molecular weight of approximately 60 kDa, consistent with the size of 59 kDa predicted from deduced amino acid sequence. Expressed and purified EasC metabolized H_2O_2, whereas a boiled control did not (Fig. 5). However, incubation of EasC directly with *N*-Me-DMAT and in the presence or absence of H_2O_2 did not lead to any new product formation.

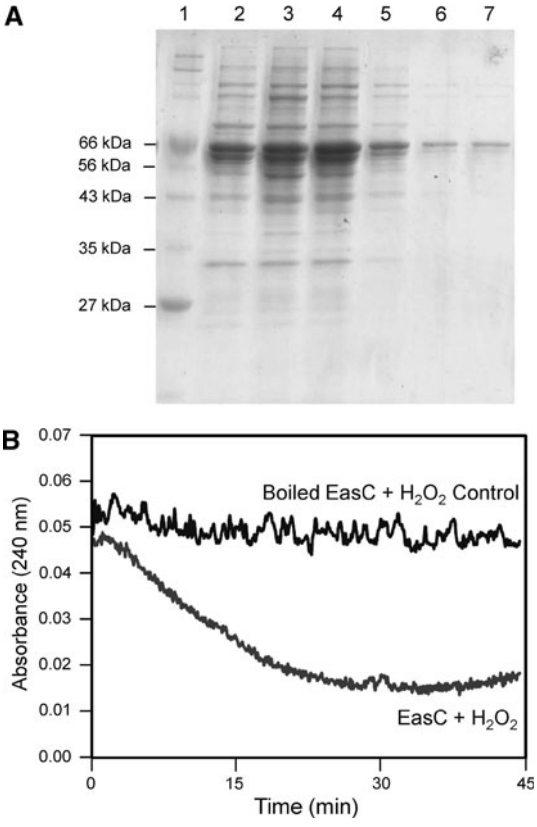

Fig. 5 Expression and activity of EasC. **a** Purification of EasC from extracts of *E. coli* overexpressing the coding sequences from *A. fumigatus easC*. Lane 1, Invitrogen Protein Marker Broad Range (2–212 kDa; relevant markers indicated); lanes 2–7, consecutive 150 mM imidazole fractions eluted from a Ni–NTA agarose column. The purest fraction (lane 7) was used for the catalase assay. **b** Catalase activity of expressed *A. fumigatus* EasC. The upper line represents 100 nM EasC inactivated by boiling prior to incubation with 10 mM H_2O_2. The lower line shows the loss in absorbance at 240 nm, corresponding to disproportionation of H_2O_2, during incubation with 100 nM purified EasC. Assay conditions were as described by Aebi (1984)

Discussion

Our data demonstrate that the catalase encoded by *easC* of the ergot gene cluster plays an essential role in the conversion of *N*-Me-DMAT into chanoclavine-I. A disruption at this point in the pathway was recently reported for *C. purpurea* strains in which the gene *ccsA* (encoding a FAD-dependent oxidoreductase that is named *easE* in *A. fumigatus*) was knocked out (Lorenz et al. 2010). The *ccsA* (*easE*) gene was disrupted in *A. fumigatus* during the course of our current study, and the Δ*easE* mutant of *A. fumigatus* also accumulated *N*-Me-DMAT. Thus, the data indicate that the catalytic activities of two different classes of enzymes, a catalase encoded by *easC* and a FAD-dependent oxidoreductase encoded by *easE*, are required to convert *N*-Me-DMAT to chanoclavine-I.

Analysis of the Δ*easC* strain by Southern hybridization demonstrated the lack of the wild-type locus and revealed the presence of two higher molecular weight fragments that hybridized with the *easC* probe. The two fragments observed in the Southern hybridization of the Δ*easC* disruptant may have been derived from either of two origins. The two bands could both be derived from the disrupted Δ*easC* locus. In this case, the approximately 15-kb fragment would result from integration of three copies of the 4,584-bp disruption construct into the 1,456-bp *Acc*I fragment of *easC*. Such a locus containing three tandem copies of the disruption construct might lose a copy of the disruption construct by intramolecular recombination. The nucleus in which recombination occurred and all nuclei derived from that nucleus, as the fungus grows, would contain a Δ*easC* locus with only two copies of the disruption construct, resulting in a fragment of 10.6 kb. Versions of the *easC* locus with any number of disruption constructs integrated would be nonfunctional. Alternatively, one of the two fragments observed in the Δ*easC* mutant may have been derived from ectopic integration of the knockout construct. It is important to note that complementation of the Δ*easC* mutant with the wild-type allele of *easC* restored ergot alkaloid production. Thus, the loss of ergot alkaloids in the Δ*easC* mutant was due to disruption at *easC* and did not result from disruption of some other gene caused by a potential ectopic integration.

Feeding exogenously supplied chanoclavine-I to the Δ*easC* mutant resulted in incorporation of chanoclavine-I into downstream ergot alkaloids. The relative proportions of downstream ergot alkaloids observed were somewhat atypical of the proportion in which these alkaloids accumulate in wild-type *A. fumigatus* (e.g., the pathway end product fumigaclavine C was present in relatively low concentrations compared to fumigaclavine A and B); however, the Δ*dmaW* mutant of *A. fumigatus* accumulated alkaloids downstream of chanoclavine-I in similar proportions

to the Δ*easC* mutant, indicating that the relative quantitative distribution of these alkaloids is a result of the experimental setup rather than some peculiarity of the Δ*easC* mutant. The data indicate that the catalase encoded by *easC* is required for the step(s) involved in converting *N*-Me-DMAT to chanoclavine-I and does not function as a general protective enzyme in *A. fumigatus*.

The presence of an apparent peroxisomal targeting sequence, amino acid residues SRL at the carboxy terminus of EasC, indicates that EasC is localized to peroxisomes. Our previous investigations of ergot alkaloid biosynthesis in *A. fumigatus* have revealed that ergot alkaloids are found in high concentration in or on conidia of the fungus (Panaccione and Coyle 2005) and are only produced in conidiating cultures (Coyle et al. 2007). EasC appears to be the only catalase of *A. fumigatus* that is both associated with conidiation and contains a peroxisomal targeting sequence. Previous investigations of the catalytically active catalases of *A. fumigatus* have identified three different catalases. Only one of these catalases (CatAp, XP_747688) was associated with conidiation as opposed to vegetative mycelium (Paris et al. 2003), but its carboxy-terminal amino acid sequence (terminating with VDH) indicates that CatAp is not peroxisomally localized. Two other proteins with catalase activity (Cat1p, XP_748550, and Cat2p, XP_747039) were expressed exclusively in mycelium of *A. fumigatus* (Calera et al. 1997; Paris et al. 2003). Cat1p lacks a C-terminal peroxisomal localizing sequence (terminating in VDH); Cat2p is a mycelial peroxidase-catalase that does appear to contain a peroxisomal localizing sequence (ARL) at its C-terminus. Searching the *A. fumigatus* genome by blastp with EasC as a query sequence reveals an additional catalase coding sequence (XP_749156), but the protein predicted from this sequence ends with YNQ, making it unlikely to be localized in peroxisomes. None of the *A. fumigatus* catalases has sequences resembling peroxisomal targeting sequence 2, as evaluated by WoLF PSORT (Horton et al. 2007). Considering the developmental association of ergot alkaloids with sporulation and the patterns of distribution and expression of catalases of *A. fumigatus*, it is reasonable that none of the other catalases of *A. fumigatus* could compensate for the loss of EasC in the Δ*easC* mutant.

The production of chanoclavine-I from *N*-Me-DMAT requires a net of four electron oxidation, which could occur as successive two-electron oxidations. Possible intermediates in the formation of the third ring of chanoclavine-I (the so-called C ring) have been investigated previously. Kozikowski et al. (1993) proposed that the C ring is formed first by hydroxylation at the benzylic carbon C10 of the prenyl group, followed by loss of water to produce a diene intermediate (Fig. 6). The diene could then undergo epoxidation, followed by decarboxylation and cyclization to form ring C. While the diene intermediate appears to be an on-pathway biosynthetic intermediate as evidenced by feeding studies (Kozikowski et al. 1993), the proposed hydroxylated or epoxide intermediates have never been observed. The finding that mutants of both *easC* and *easE* accumulate the same intermediate suggests that these two enzymes, each with different catalytic activities, act in a coordinated fashion. If these enzymes acted sequentially, it would be expected that an intermediate other than *N*-Me-DMAT would accumulate in one of the mutant strains. Nonetheless, the possibility that the enzymes act sequentially and that the latter-acting enzyme has a substrate that is unstable to the point that it cannot be detected in our assays, cannot be excluded. In either case, the activities of both the catalase and the FAD-containing oxidoreductase would be required for the oxidation of *N*-Me-DMAT to chanoclavine-I.

Although EasC is required for incorporation of *N*-Me-DMAT into downstream ergot alkaloids, EasC purified from a heterologous expression system does not appear to turnover *N*-Me-DMAT in vitro. EasC was shown to decompose H_2O_2 in vitro, suggesting that the heterologous enzyme is properly folded and contains the required cofactors for catalase activity. It is possible that the ergot alkaloid pathway requires disproportionation of H_2O_2, and that the sole function of EasC is to remove H_2O_2 derived from the oxidative activities of EasE. Notably, FAD-containing oxidases like EasE can generate H_2O_2 that is decomposed to water and oxygen by a partner catalase (Geissler et al. 1986; Das et al. 2010).

The accumulation of a common intermediate in Δ*easC* and Δ*easE* mutants indicates that EasC and EasE work together; however, the nature of their direct interaction, if any, is not yet known. A specific interaction of the EasC catalase with EasE (a potential H_2O_2 generator) could be required for proper functioning of both proteins. However, the respective amino acid sequences have localization sequences that make it difficult to assess how they would interact. EasC contains a classic C-terminal peroxisomal localization sequence (carboxy-terminal residues SRL), whereas EasE lacks a peroxisomal localization sequence at its carboxy terminus and has amino terminal sequences that are a good match for a signal peptide.

To explore these issues in an in vitro assay, extensive efforts were made to heterologously express EasE in both *E. coli* and *Saccharomyces cerevisiae*. Unfortunately, the heterologously expressed protein was produced at nearly undetectable levels (data not shown). Moreover, UV absorption spectra of the crude protein at 446 nm indicated that the required flavin cofactor was not present, which means that this enzyme cannot be catalytically active. Not surprisingly, in vitro assays of EasE, or EasE in combination with EasC, also failed to turn over the *N*-Me-DMAT substrate. Efforts are ongoing to obtain catalytically active, holo-EasE for in vitro enzymatic assays to more fully

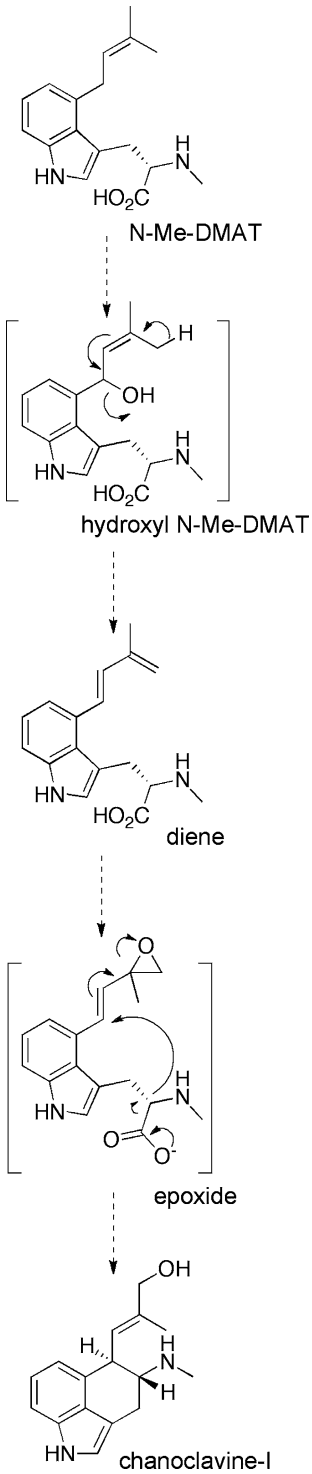

Fig. 6 Scheme for conversion of *N*-Me-DMAT to chanoclavine as proposed by Kozikowski et al. (1993). Hypothetical intermediates are indicated in brackets

explore the potential interaction of EasC and EasE in the ergot biosynthetic pathway that is suggested by the in vivo disruption experiments.

Our data demonstrate that EasC and EasE are required for the conversion of *N*-Me-DMAT to chanoclavine-I; whether these two enzymes are sufficient for this catalysis or if additional enzymes are required has not yet been determined. Analysis of ergot alkaloid gene clusters from several fungi that produce ergot alkaloids indicates that there are no more shared cluster genes that have not been functionally analyzed. Thus, if additional enzymes are required, those enzymes would have to be encoded outside of the ergot alkaloid gene clusters, which would be atypical among fungal secondary metabolite gene pathways. Our laboratories are currently working on genetic and biochemical approaches to test whether EasE and EasC are sufficient for the conversion of *N*-Me-DMAT to chanoclavine-I. A deeper understanding of the ergot alkaloid pathway, along with the capacity to engineer it at different steps, may have ramifications in agriculture, where ergot alkaloids adversely affect animal production, and also in medicine, where modification of the alkaloid structure may productively modulate the strong pharmacological activities of these compounds.

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