The Key Ergot Alkaloid Intermediate Chanoclavine-I Produced in Yeast (*Saccharomyces cerevisiae*) by the Combined Action of EasC and EasE from *Aspergillus japonicus*

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Running Head: Chanoclavine-I Production in Yeast

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Filamentous fungi produce a range of bioactive molecules, among them the ergot alkaloids. These compounds have been intensely studied for decades, mainly due to their deleterious effects in contaminated food and feeds, but also for their beneficial pharmaceutical and agricultural applications. The genes encoding the various ergot alkaloid pathways are naturally arranged in genomic clusters, which share a high degree of similarity among the fungal producers. They produce the same initial intermediates up to chanoclavine-I and chanoclavine aldehyde, before branching off towards species specific ergot alkaloids. The role of individual gene products of the pathway have been broadly elucidated, although for the conversion of Me-DMAT to chanoclavine I, the predicted roles of EasC and EasE have been based on complementation in closely related fungi. Difficulties of obtaining purified, active EasE has so far prevented confirmation by *in vitro* biochemical studies. In this study we reconstituted the chanoclavine-I pathway in yeast (*S. cerevisiae*), taking advantage of the recent publication of an ergot alkaloid cluster from *A. japonicus*. We demonstrate that EasC and EasE are both necessary and sufficient for the production of chanoclavine-I. In addition, we review some of the challenges involved in expressing EasE and suggest a requirement for folding and disulphide bridge formation via the secretory pathway.

Ergot alkaloids (EA) belong to a diverse group of natural compounds with a range of biological activities that have important applications in medicine and agriculture (1-3). Some of these compounds have a notorious neurological effect on humans, possibly due to the structural similarity of these compounds to neurotransmitters like serotonin and dopamine (4). EAs are produced by a variety of plant associated fungi, mainly of the genera *Claviceps* and *Aspergillus*. The production of ergot alkaloids has been linked to biosynthetic gene clusters found in several species, first in *Claviceps purpurea* (5) and later in *Aspergillus fumigatus* (6,7), *Neotyphodium lolii* (8) and others (see (3) for review).

The ergot alkaloids can be divided into three classes, clavines, ergoamides, and ergopeptines, depending on the substitutions found on the basic ergoline scaffold. The biosynthetic pathways leading to various ergot alkaloids have been partially elucidated, and the early steps leading to the common biosynthetic intermediate chanoclavine aldehyde are identical. After biosynthesis of chanoclavine aldehyde the biosynthetic intermediates diverge (Fig. 1). Hence, in *C. purpurea* the pathway continues via agroclavine to ergotamine and in *A. fumigatus* via festuclavine to the fumigaclavines (3).

The first step of the common pathway is the electrophilic aromatic addition of dimethylallyl-pyrophosphate (DMAPP) to the 4 position of tryptophan to form dimethylallyl-tryptophan (DMAT). The reaction is catalysed by a prenyl transferase, DmaW (fgaPT2 in *A. fumigatus*) (6,9). The second step, the methylation of DMAT to form Me-DMAT, is catalysed by the methyltransferase EasF (fgaMT in *A. fumigatus*) (10).

While the biosynthesis of Me-DMAT is well understood, the mechanistic basis behind the conversion of Me-DMAT to chanoclavine-I remains unclear. The conversion of Me-DMAT to chanoclavine-I was investigated by gene disruption and complementation studies in *C. pupurea* and *A. fumigatus*: Lorenz and co-workers (11) used a mutated *C. purpurea* strain P1 to show that deletion of the easE_Cp (ccsA) gene abolished production of chanoclavine-I and any downstream products. Instead an accumulation of Me-DMAT was seen, indicating a block in the pathway after this intermediate. Alkaloid biosynthesis could be restored by expressing a GFP fusion construct of the easE_Cp gene. Analogously, Goetz and co-workers (12) disrupted the easC_Af gene in *A. fumigatus*, and also observed accumulation of Me-DMAT and the absence of downstream products. Furthermore, a similar pattern was observed when easE_Af was disrupted, in concordance with the *C. purpurea* results (above). In both of the easE and easC deletion strains, the alkaloid pathway could be restored by re-introduction of the corresponding wild type allele. Most recently Ryan and co-workers (13) transferred

part of the *A. fumigatus* EA cluster, comprising the four genes dmaW, easF, easC, and easE, into *A. nidulans*, a fungus known as a non-producer of any EA. This partial cluster conferred the ability to produce chanoclavine-I, further suggesting that EasE and EasC are sufficient for the conversion from Me-DMAT. However, the involvement of enzymes from cryptic EA clusters in *A. nidulans* cannot be excluded (14).

Interestingly, EasC_Af contains a C-terminal amino acid motif (SRL), which is a classic type 1 peroxisomal targeting signal (PTS1) (15). Similar signals are found in many EasC homologues of *Aspergillus spp*., but not in *Claviceps spp*. EasE_Af, on the other hand, has an N-terminal sequence which strongly resembles a signal peptide for entering the ER and secretory pathway. However, the implication of these localisation signals remains unclear, particularly in light of the apparent co-operation between EasC and EasE.

The EasC proteins have similarity to peroxisomal catalases (6, 7, 16). EasC_Af (the easC_Af gene product) was purified after expression in *E. coli*, and *in vitro* catalase activity of EasC_Af was shown using H_2O_2 as substrate (12). However, when the enzyme was incubated with Me-DMAT, no new product was detected. Extensive efforts were made to produce active EasE_Af from *E. coli* or *S. cerevisiae* (12). However, in all cases, incubation of EasE with Me-DMAT, or of EasE with Me-DMAT and EasC, failed to produce any new product. Therefore, biochemical studies to understand the transformation of Me-DMAT to chanoclavine-I have not been possible.

As an alternative approach to address some of the open questions regarding the early EA pathway we undertook the reconstitution of the chanoclavine-I pathway in yeast (*S.cerevisiae*). Yeast is the work horse of eukaryotic gene expression and is easily amenable to genetic manipulation (17, 18). Heterologous genes are generally well expressed, and in particular for proteins which might require an ER-associated folding, the yeast cell seems to

be a suitable host. The immediate goal was to assess whether, despite the reported difficulties with heterologous EasE expression, we could engineer a yeast for *de novo* production of chanoclavine-I. This would facilitate further study into the roles of EasC and EasE in the intriguing biochemical conversion from Me-DMAT into chanoclavine-I, and eventually pave the way for heterologous production of ergot alkaloids on a commercially relevant scale.

MATERIALS AND METHODS

DNA and protein sequence analysis. Computer-aided sequence analysis was done using Vector NTI 9.1.0 software (Invitrogen Corp. 2004) and the free online software FGENESH (http://linux1.softberry.com/berry.phtml), GENSCAN (http://genes.mit.edu/GENSCAN.html), and the NCBI server (http://www.ncbi.nlm.nih.gov). Signal peptides were predicted using the SignalP 4.0 tool (19).

Preparation and cloning of genes in yeast expression vectors. Synthetic genes, codon optimized for expression in yeast, were manufactured by DNA2.0 Inc., Menlo Park, CA, USA or GeneArt AG, Regensburg, Germany. All sequences were derived from *A. japonicus*, *A. fumigatus*, or *C. purpurea*. The genes encode the amino acid sequences, plus a translation stop codon, of DmaW_Af (acc. no. XP_756141), DmaW_Cp (acc. no. CAB39314), EasF_Af (acc. no. XP756143), EasC_Af (acc. no. XP756140), EasE_Af (acc. no. XP756142), and EasE_Cp (acc. no. CAB39328). The sequences DmaW_Aj1, EasF_Aj, EasC_Aj, and EasE_Aj were predicted, based on the genomic DNA sequence of the cycloclavine gene cluster of *A. japonicus* (20). All genes were synthesized with the DNA sequence AAGCTTAAA, containing a HindIII restriction recognition site, at the 5´-end and with a SacII recognition site at the 3´-end (CCGCGG), and these sites were used for cloning. All PCR primers used for sub-cloning contained these sequences. Standard PCR conditions were

used according to manufacturer's recommendations (BioRad iProof High Fidelity DNA polymerase, Cat. #172-5302)

Gene dmaW_Aj3 was prepared by PCR using dmaW_Aj1 as template, and dmaW_Aj2 was prepared by sequential extension PCR, using dmaW_Aj3 as template, thus adding the second exon in two steps (Table S1). The easC_Aj version without C-terminal PTS1 signal was prepared by PCR amplification of the coding sequence (CDS) without the 9 bps before the stop codon. The easE_Aj N-terminal truncation (easE_Aj -N sig.) was done by PCR, amplifying the CDS without the first 87 bps. The forward PCR primer inserted an alternative ATG translation start site. The fusion of an N-terminal signal peptide from Pdi1 to the N-truncated EasE_Aj was done by overlapping extension PCR, fusing the two amplicons to give Pdi1-EasE_Aj (Table S2). The CDS of the native yeast genes pdi1 (acc. no. D00842), ero1 (acc. no. NM_001182493), and fad1 (acc. no. NM_001180104) were amplified from genomic DNA by PCR.

For expression, all genes were cloned into expression vectors based on pRS313, pRS315, and pRS316 (21). These vectors had been provided with a new multi-cloning site (MCS) linker, inserted between the two PvuII sites. The basic design of the MCS was SrfI-AscI-BglII-HindIII-SfiI(a)-SfiI(b)-SacII-SphI-AscI-SrfI (22). The linker allowed cloning of promoter sequences into BglII and HindIII, and terminators into SacII and SphI restriction sites to create yeast expression cassettes. Promoters and terminators were amplified from yeast genomic DNA by PCR (Table S3) for preparing three expression cassettes containing 1) a Gpd1 promoter and a Cyc1 terminator (G/C), 2) a Pgk1 promoter and an Adh2 terminator (P/A), and 3) a Cup1 promoter and an Adh1 terminator (C/A). The new expression vectors were named pRS31X-G/C, pRS31X-P/A, and pRS31X-C/A, where X designates 3, 5, or 6. All genes used in this study (Table 1) were cloned into expression cassettes of these vectors.

Construction and integration of yeast gene expression cassettes. Constructs for integration were prepared for the integration sites YORWΔ22 and YPRCΔ15 (23), and cloned in unique EcoRI and HindIII sites of a pUC19 vector backbone. The homologous regions were constructed from two PCR fragments, which were then combined by overlapping extension PCR. The PCR primers introduced the restriction sites AscI and NotI between the two original fragments, and SbfI sites at the outer ends. The KanMX cassette, flanked by loxP sites, was excised from pUG6 (24) and inserted into NotI. Two expression cassettes (described above) were inserted into the AscI site. The first cassette was amplified by PCR, changing one AscI site to an MluI. After cloning this fragment, the second cassette was inserted into the single regenerated AscI site. The entire construct was released by SbfI and used for integration. This approach was used to first integrate dmaW_Aj2 (G/C cassette) and easF_Af (P/A cassette) into the yeast genome at the YORWΔ22 site. After excision of the KanMX marker (24), the easC_Aj (G/C cassette) and easE_Aj (P/A) cassette were integrated into the YPRCΔ15 site. Yeast transformations were done using the LiAc method (25).

Yeast strain and culture conditions. The host used in this study was a *S. cerevisiae* strain with the genotype MATα, his3Δ1, leu2Δ0, lys2Δ0, trp1Δ0, ura3Δ0. Engineered yeast strains were grown in standard SC broth with 2% glucose, minus leucine and histindine (ForMedium, Hunstanton, U.K.). When appropriate, $CuSO_4$ was added to a final concentration of 300 µM for induction of gene expression. Cultures were grown with constant shaking at 30°C for 72 hours in 250 ml shake flasks containing 25 ml medium.

Analytical procedures. For analysis yeast cultures were spun down for 10 min at 1000 × g. The pellet and the supernatant were separated. Without further purification, 5 µl of supernatant were injected in a UPLC-TOF (Waters Acquity^{TM} Ultra Performance LC, Waters, Milford, Mass., USA) coupled to a micrOTOF-Q II (Bruker Daltonik GmbH, Bremen, Germany). Stationary phase column was an Acquity UPLC® Bridged Ethyl Hybrid

(BEH) C18; 1.7 µm; 2.1×100 mm. Liquid chromatography used mobile phases of H_2O + 0.1% formic acid (A), and acetonitrile + 0.1% formic acid (B), in a linear gradient of 1% to 100% B in 5 min. The column was washed for 1 min in 100% B, and then equilibrated for 1.5 min in 1% B. Detection of compounds was done by a photo diode array using the following parameters: λ range: 210 nm to 500 nm. Resolution: 1.2 nm. Sampling rate: 5 points/s. ELSD parameters: gain 50, gas pressure 40 psi, nebulizer mode: heating, power level: 80%, drift tube: 80°C. TOF parameters: Source: End Plate Offset: -500V. Capillary: -4500V. Nebulizer: 1.6 bar. Dry gas: 8.0 l/min. Dry temperature 180°C. Scan mode: MS Scan. Mass range: from 80 to 1000 m/z.

Preparative procedures. For compound purifications yeast cultures were spun down for 10 min at 1000 × g. The supernatant was adjusted to pH=10 with 10M NaOH and extracted by liquid/liquid extraction with an equal volume of ethyl acetate. The crude extract was dried under vacuum and reconstituted with dimethyl sulfoxide (DMSO) to a concentration of 100 mg/ml and then purified on a preparative HPLC system (Waters, Milford, Mass, USA). Stationary phase was an XBridge™ preparative C18, 5 µm, 19×250 mm column. Liquid chromatography used mobile phases of H_2O + 0.1% trifluoroacetic acid (A), and acetonitrile + 0.1% trifluoroacetic acid (B), in a linear gradient of 1% to 30% B in 40 min. The column was washed for 5 min in 100% B, and then equilibrated for 5 min in 1% B. Fractions were collected every 2 min and analyzed as above. Fractions containing the purified analyte were pooled and dried under vacuum.

RESULTS

Prediction of dmaW coding sequences. An ergot alkaloid gene cluster was recently identified in *A. japonicus* (20), one of several fungal species currently being investigated for their capacity to produce molecules of potential commercial importance (26-28). *A. japonicus*

191 was previously reported to produce the EA cycloclavine (29), an EA of the clavine group,
192 and the genome sequence of the cluster displays high homology to EA clusters of *Claviceps*
193 *spp.* and *Aspergillus spp.* This cluster therefore provided an interesting alternative for
194 studying the chanoclavine-I pathway. The putative coding sequences (CDS) of dmaW, easF,
195 easC, and easE were predicted, using free online gene prediction software and by alignment
196 to homologues in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Analysis of
197 the dmaW sequence in the *A. japonicus* genome revealed two different CDS predictions: one
198 prediction was for a single open reading frame of 1602 bps, corresponding to a 534 amino
199 acid protein, whereas another prediction, with the same translation start codon, was for two
200 exons of 1287 bps and 150 bps separated by an 85 bps intron, corresponding to a 479 amino
201 acid protein. Both of these predictions were slightly longer than similar DmaW enzymes
202 found in the GenBank database, where the length of DmaW homologues were in the range of
203 435-465 amino acids. Moreover, a multiple protein alignment in GenBank, using either of the
204 two *A. japonicus* DmaW predictions as query, showed a lack of homology beyond approx.
205 428 amino acids, i.e. beyond the predicted first exon. A somewhat similar situation was seen
206 for the two entries of *A. fumigatus* DmaW (Fig. S1). Hence, we decided to test, not only the
207 two predictions encoding 534 amino acids (dmaW_Aj1) and 479 amino acids (dmaW_Aj2),
208 but also a shorter version encoding 429 amino acids (dmaW_Aj3), corresponding to the
209 predicted first exon. For easF, representing the next step in the pathway, we tested the
210 homologues easF_Af and easF_Aj, from *A. fumigatus* and *A. japonicus,* respectively.

211 **Me-DMAT production in yeast.** To analyse the biosynthesis of chanoclavine-I we first
212 constructed a yeast strain designed to produce the chanoclavine-I precursor Me-DMAT. For
213 this, plasmids were constructed with synthetic, yeast codon optimized genes corresponding to
214 each of the three dmaW predictions described above, as well as the dmaW_Cp (AJ011963)
215 and dmaW_Af (XM_751048) (Table 1). All genes were tested by co-expression with a codon

optimized easF_Aj gene. The easF_Aj gene was cloned in pRS315-C/A, and each dmaW gene was cloned in the pRS316-C/A, allowing expression from the Cup1 promoter. Combinations of easF_Aj and each of the dmaW genes were then introduced in yeast and expressed by inducing the Cup1 promoter. After 72 h of growth at 30°C, the culture supernatants were analysed by LC-MS, and the expected mass to charge ratio (m/z) of DMAT (m/z = 273.159 +/- 0.01) and Me-DMAT (m/z = 287.175 +/- 0.01) were extracted from the total ion chromatograms. The area under the corresponding peaks was calculated and compared, showing production of both DMAT and Me-DMAT in the strains expressing dmaW_Aj2, dmaW_Aj3, and dmaW_Cp, but essentially no production with dmaW_Aj1 or dmaW_Af (Fig. 2). We suspect that dmaW_Aj1 is likely to be an incorrect CDS prediction. We also noted that, compared to the homologue used in a previous study (6), dmaW_Af has an 8 amino acid deletion and speculate that the sequence of dmaW_Af may also be an incorrect prediction. (Fig. S1).

DMAT accumulated in each of the three strains producing Me-DMAT. This indicated relatively poor activity of the methyl transferase EasF_Aj. We therefore tested the *A. fumigatus* homologue EasF_Af, and with this enzyme almost all of the DMAT was converted to Me-DMAT in a strain co-expressing dmaW_Aj2 (Fig. 2). Hence, for further studies the combination of dmaW_Aj2 and easF_Af was integrated into the yeast genome. The new strain was used to purify Me-DMAT, and the compound was analysed by ^{1}NMR to confirm the identity (Fig. S2a).

Chanoclavine-I production in yeast. Having established the production of Me-DMAT in yeast, we next addressed the conversion of this compound into chanoclavine-I. Preliminary results in our laboratory had shown poor expression of a C-terminal GFP-fusion construct of EasE_Aj, which is consistent with the reported difficulties of purifying the corresponding enzyme, EasE_Af, from *A. fumigatus* (12). Therefore, for this study, we tested several EasE

241 homologues from *A. japonicus*, *A. fumigatus*, as well as from *C. purpurea*. To investigate the
242 hypothesis that EasC and EasE are both required for this conversion (12, 13) we expressed
243 easC_Aj, in combination with each of the three different easE homologues, in the background
244 of the Me-DMAT producing yeast (see above). Each easE homologue was cloned into
245 pRS313-G/C, and easC_Aj into pRS315-P/A. When combining easC_Aj and easE_Aj we
246 saw the appearance of a new compound with a retention time of 4.3 min, which had the
247 expected m/z of chanoclavine-I (m/z = 257.165 +/- 0.01). The compound co-eluted with a
248 compound of identical mass found in an *A. japonicus* mycelium extract (Fig. 3). The
249 compound at 4.3 min was purified, and the identity was confirmed by ^{1}H NMR to be
250 chanoclavine-I (Fig. S2b). Chanoclavine-I was not detected in strains expressing easC_Aj in
251 combination with either easE_Af or easE_Cp, indicating that the *A. fumigatus* and *C.*
252 *purpurea* homologues used in this study may not be functional in yeast. In strains expressing
253 only an easE or an easC homologue no chanoclavine-I was detected, supporting the
254 hypothesis that both easE and easC are required for its biosynthesis. The combination of
255 easC_Aj and easE_Aj was integrated into the genome of the Me-DMAT producing strain (see
256 above), and the resulting strain was used to purify chanoclavine-I, which was again
257 characterized by ^{1}H NMR.

258 **Peroxisomal targeting signal is not needed in yeast.** As noted by Goetz and co-workers
259 (12) EasC_Af has a classical PTS1 peroxisomal targeting sequence, the tri-peptide SRL, at its
260 carboxy-terminal end similar to PTS1 signals commonly found in yeast (15). One such
261 putative signal, ARL, is also present in the *A. japonicus* EasC_Aj sequence, as well as in
262 other homologues within the *Aspergillus* genus. However, in the *Claviceps* genus no obvious
263 PTS1 signal is found and the *C. purpurea* EasC homologue instead has a C-terminal IVE,
264 which has no resemblance to the PTS1 consensus sequence. Assuming the EA enzymes from
265 these fungi have similar function, and therefore localization, this is somewhat puzzling and

we therefore wondered if the PTS1 of *Aspergilli* is important for function. Hence, we prepared an EasC-Aj version in which the ARL triplet was deleted. A set of strains were prepared expressing the integrated DmaW_Aj2 and EasF_Af, together with EasE_Aj (in pRS313-G/C) and either the new EasC-Aj -PTS1 version (in pRS315-P/A) or the original full length EasC_Aj (in pRS315-P/A). To our surprise we saw no major difference in the ability to produce Me-DMAT and chanoclavine-I. In fact, the version without the putative PTS1 sequence resulted in slightly higher concentrations of Me-DMAT and chanoclavine-I. (Fig. 4)

N-terminal signal is crucial for EasE function. A common feature observed for the EasE homologues is a high number of hydrophobic amino acids at the N-terminal end, which is typically associated with signal peptides for the secretory pathway. Analysis of the amino acid sequences of EasE_Aj, EasE_Af, and EasE_Cp, using the online SignalP server (19), confirmed this notion by predicting signal peptides in all three enzymes. A putative cleavage site in EasE_Aj was predicted after pos. 29, i.e. between A and V. We used this information to prepare a truncated version of the enzyme, which lacked the N-terminal sequence, and expressed it from the pRS313-G/C plasmid, in the Me-DMAT producing strain, together with EasC_Aj (in pRS315-P/A). The N-truncation seriously impaired the functional expression of EasE_Aj, and essentially no chanoclavine-I was detected using this enzyme. Instead, accumulation of the precursor compound Me-DMAT was observed (Fig. 4).

To further evaluate the function of the putative signal peptide we prepared a version of EasE_Aj, in which we replaced the N-terminal 31 amino acids (predicted signal peptide including the cleavage site) with the known signal peptide from the native yeast Pdi1 protein. DNA sequences were fused to encode the N-terminal 24 amino acids from Pdi1, which includes the cleavage site, followed by the EasE_Aj peptide from pos. 32 (Table S2). The fusion protein was tested as described for the truncated version. When expression was compared to the original EasE_Aj approximately half the chanoclavine-I production level

was observed, indicating that the Pdi1 signal peptide provided functionality to the enzyme (Fig. 5). This was taken as an indication that for proper function the EasE needs to enter the secretory pathway. It is not clear why the yields were lower when the Pdi1 sequence was used.

Pdi1 or Ero1 overexpression seems to improve the function of EasE. We speculated that the importance of the N-terminal sequence is linked to a requirement for disulphide bond formation as part of the protein maturation process, which would happen during ER associated translation. EasE_Aj has a total of 9 cysteines which could potentially form disulphide bridges, and the majority of these cysteines are highly conserved among EasE homologues (Fig. S3). For a preliminary evaluation of the importance of these cysteines we mutated the first seven of these individually, replacing them with alanine. All of these mutations resulted in complete loss of function (data not shown). The native yeast enzymes Pdi1 and Ero1 are known to have a function in the formation of disulphide bonds in ER (see (30) for review). Hence, a pair of chanoclavine-I producing strains were prepared in which we over-expressed one of the two genes, either pdi1 or ero1, to test whether this would have any impact on the production of chanoclavine-I. Over-expression of pdi1 (in pRS313-G/C) resulted in an approx. 50% increase in chanoclavine-I production, whereas ero1 over-expression (in pRS313-C/A) caused an almost 3 fold increase (Fig. 6), supporting a possible involvement of disulphide bond formation in proper EasE folding.

Fad1 overexpression seems to improve the function of EasE. EasE has previously been described as a flavin adenine dinucleotide (FAD) dependent reductase or dehydrogenase, and in support of this the structural similarity to the class of berberine bridge enzymes (BBE) has previously been pointed out (11). In plants the BBE enzyme is involved in alkaloid biosynthesis, and it has been associated with transport from ER to the vacuole (31). BBE was shown to bi-covalently bind a flavin co-factor (32) and the binding site, involving a histidine

316 and a cysteine, seems to be conserved in EasE_Aj and other EasE homologues (Fig. S4).
317 With ample evidence that EasE is FAD dependent, we speculated whether an increased
318 supply of this co-factor would directly improve the function of EasE. An attempt to increase
319 the supply via the growth medium showed no effect on chanoclavine-I production (data not
320 shown), so instead we cloned the native yeast fad1 gene, which encodes the enzyme
321 responsible for the synthesis of FAD from flavin mono nucleotide (FMN). Over-expression
322 of this gene, in a strain harbouring the chanoclavine-I pathway, led to a 2.5 fold increase in
323 chanoclavine-I production (Fig. 6) which we interpreted as an effect of improved co-factor
324 supply.

325

326 **DISCUSSION**

327 Heterologous expression in yeast of the four enzymes DmaW_Aj2, EasF_Af, EasC_Aj, and
328 EasE_Aj led to production of chanoclavine-I, demonstrating that yeast is a suitable host for
329 producing ergot alkaloids. This work also strongly suggests, in accordance with previous
330 published studies, that EasC and EasE are solely responsible for the conversion of Me-
331 DMAT to chanoclavine-I without the involvement of other EA enzymes. Secondary
332 metabolism in *S. cerevisiae* is quite limited and it seems unlikely that any yeast enzyme
333 would be involved in the highly specialized metabolic process of ergot alkaloid biosynthesis.
334 The production of chanoclavine-I depended on the expression of EasE from *A. japonicus*,
335 whereas EasE from *A. fumigatus* or *C. purpurea* were not active when expressed in yeast. We
336 speculate that the predicted coding regions of easE_Af and easE_Cp, along with dmaW_Aj3,
337 could be incorrect (see below, and also Fig. S3 and S5), and certainly the prediction of intron
338 and exon sequences in filamentous fungi is still a challenge.

Our results support the notion that EasE and EasC co-operate to produce chanoclavine-I, but it is still not clear how or where this process occurs. As shown here the putative PTS1 signal of EasC_Aj was not crucial for function, and it is possible that even with the PTS1 signal the natural cellular distribution involves multiple locations, as seen for some native yeast proteins (33, 34). The N-terminal signal of the EasE, on the other hand, was clearly crucial for function, and the fact that a native yeast signal peptide from Pdi1 partially restored function indicates that EasE contains a genuine ER targeting signal. This would suggest that folding and disulphide bond formation in the ER is needed for proper maturation of EasE and that it passes through the secretory pathway before, possibly, joining up with EasC. Further cellular localization studies might elucidate this puzzle.

The case for oxidative folding of EasE in ER is supported by the observed increase in chanoclavine-I production after over-expression of either of the two key enzymes in the disulphide bridge formation machinery. Ero1 is a sulfhydryl oxidase responsible for generating disulphide bonds that are passed on to Pdi1, which in turn oxidizes the cysteines of newly translated proteins (reviewed in 30, 35, 36). The EasE family contains several highly conserved cysteines that would be available for oxidation (see below), although one cysteine is likely to be involved in binding the FAD co-factor.

Interestingly, a multiple sequence alignment of the EasE_Aj and its closest homologues showed some unexpected dissimilarity regarding EasE_Af and EasE_Cp (Fig. S5). The first approx. 130 amino acids of EasE_Af showed no similarity to the consensus sequence, whereas EasE_Cp seemed to be completely lacking the N-terminal domain. An earlier prediction of EasE_Cp (11) suggested a 483 amino acid peptide derived from two exons. A newer GenBank entry (acc. no. JN186799), however, predicts a third exon at the 5-end and encodes a 595 amino acid protein including a signal peptide, as predicted by the SignalP model (19). The study (11) reported complementation of an easE_Cp gene knock-out after

integration of a PCR fragment comprising the same easE gene including its native promoter region. We speculate that this PCR fragment may by chance have included the first exon, allowing correct splicing and, hence, full complementation. Complementation by re-integration into the easE locus was excluded, since integration at the niaD locus was confirmed by Southern hybridization. A similar approach was used for studying the easE gene in *A. fumigatus* (12). The native easE gene was first disrupted, which abolished function, and the WT sequence was then re-integrated along with 1445 bps 5'-flanking sequence. The inclusion of upstream sequence would allow the fungus to splice the gene correctly, and any divergence between the mature mRNA and the predicted CDS of easE_Af would not have been detected. We analysed the genomic region of *A. fumigatus* chromosome II using an online intron prediction model, which suggested an 1809 bps easE_Af coding sequence, encoding a protein of 602 amino acids. This newly predicted protein shows very high sequence similarity to other EasE proteins in the multiple alignment (Fig. S3).

We show here that EasC and EasE are responsible for the conversion of Me-DMAT to chanoclavine-I. Moreover, we demonstrate that the biosynthetic pathway for chanoclavine-I, the central biosynthetic precursor for all ergot alkaloids, can be transferred to the industrially important host, *S. cerevisae*. This discovery will greatly facilitate further genetic and metabolic engineering of the ergot alkaloids, which have a broad range of pharmaceutical and agrochemical uses. Our yeast strain may serve as a starting point to pave the way for commercial scale production of known or novel ergot alkaloids. Although much work is still pending toward a full understanding of the biochemical reactions involved for chanoclavine-I production, the insights that we have obtained in this study should facilitate more successful biochemical analyses of these reactions. In addition, the impact of cellular localization of the enzymes and the steps involved in the maturation process until they reach their final destination, presents new challenges for further studies.

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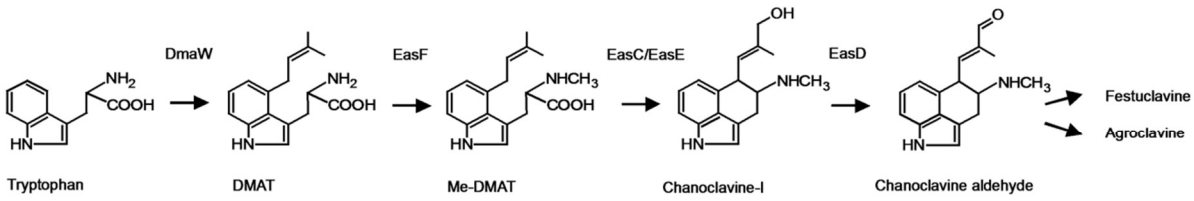

FIG 1 Biosynthetic pathway to chanoclavine-I and chanoclavine aldehyde, starting from tryptophan and DMAPP (not shown). Chanoclavine aldehyde is considered the branch point in the pathway to different ergot alkaloids via intermediates like agroclavine and festuclavine.

TABLE 1 Genes used in this study*

CDS name	Source	Amino acids
dmaW_Aj1	WO2012/116935	534
dmaW_Aj2	WO2012/116935	479
dmaW_Aj3	WO2012/116935	428
dmaW_Af	XM_751048	451
dmaW_Cp	AJ011963	448
easF_Aj	WO2012/116935	340
easF_Af	XM_751050	339
easC_Aj	WO2012/116935	510
easC_Af	XM_751047	520
easC_Aj -PTS1	WO2012/116935	507
easE_Aj	WO2012/116935	622
easE_Af	XM_751049	628
easE_Cp	AJ011965	483
easE_Aj -N sig.	WO2012/116935	594
pdi1/easE_Aj	D00842/WO2012/116935	615
pdi1_Sc	D00842	522
ero1_Sc	NM_001182493	563
fad1_Sc	NM_001180104	306

* The gene names are followed by a two-letter code to indicate species of origin: Aj: *A. japonicus*; Af: *A. fumigatus*; Cp: *C. purpurea*; Sc: *S. cerevisiae*. All genes were synthesized with yeast codon optimization, except for pdi1_Sc, ero1_Sc, and fad1_Sc, which were prepared by PCR on yeast genomic DNA. Synthesis and PCR primer design was based on the named sources to give the corresponding proteins, spelled with an initial capital letter, of the listed length.

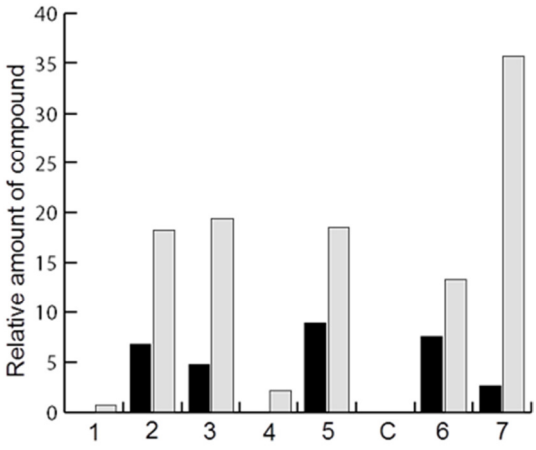

FIG 2 Relative production of DMAT (black bars) and Me-DMAT (grey bars) were analysed in strains co-expressing EasF_Aj in combination with DmaW_Aj1 (1), DmaW_Aj2 (2), DmaW_Aj3 (3), DmaW_Af (4), or DmaW_Cp (5). Expression of DmaW_Aj1 and DmaW_Af resulted in only small amounts of compounds compared to the other three DmaW homologues. Similarly, DmaW_Aj2 was co-expressed with the two homologues EasF_Aj (6) or EasF_Af (7) and relative DMAT and Me-DMAT amounts were analysed. The EasF_Af resulted in more than double the amount of chanoclavine-I compared to EasF_Aj. The control strain (C) carried an empty plasmid with no DmaW expression. The vertical axis shows arbitrary units based on area under the HPLC peak.

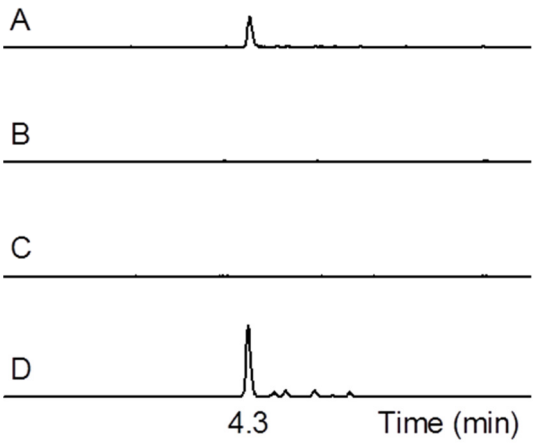

FIG 3 Extracted ion chromatograms corresponding to chanoclavine-I ($[M+H]^+$ = 257.16) of yeast strains co-expressing dmaW_Aj2, easF_Af, and easC_Aj in combination with one of three different homologues, easE_Aj (A), easE_Af (B), or easE_Cp (C). Only the strain co-expressing easE_Aj produced chanoclavine-I, whereas no chanoclavine-I was detected in strains expressing either easE_Af or easE_Cp. Retention time and m/z of (A) corresponded to a chanoclavine-I reference extracted from *A. japonicus* mycelium (D).

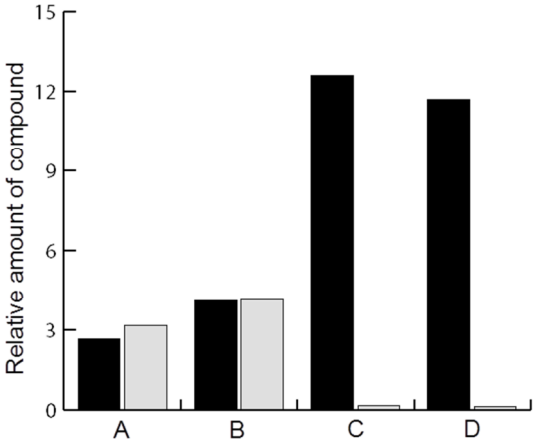

FIG 4 Relative production of Me-DMAT (black bar) and chanoclavine-I (grey bar) was analysed, of a Me-DMAT producing strain, co-expressing EasC and EasE proteins with different localization signals. Wild type EasE_Aj was co-expressed in combination with either wt EasC_Aj (A) or EasC_Aj -PTS1which has no PTS1 (B). Removal of the PTS1 tripeptide resulted in a slight increase of Me-DMAT and chanoclavine-I. However, when an N-terminally truncated EasE_Aj –N sig. was co-expressed with either wt EasC_Aj (C) or EasC_Aj -PTS1 (D), production of chanoclavine-I was essentially abolished. The loss of function, due to the N-terminal truncation of EasE_Aj, resulted in increased accumulation of the precursor Me-DMAT (C and D compared to A and B). The vertical axis shows arbitrary units based on area under the HPLC peak.

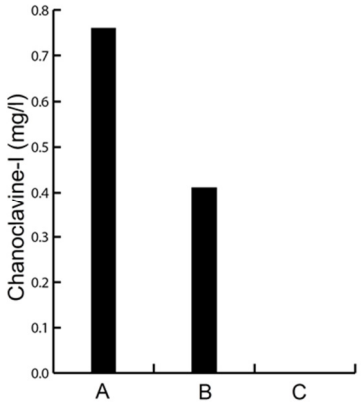

FIG 5 Concentration (mg/l) of chanoclavine-I, was measured in the growth medium, of a strain expressing DmaW_Aj2, EasF_Af, and EasC_Aj after co-expression of different versions of EasE. Expression of the wt EasE_Aj (A) resulted in production of 0.75 mg/l chanoclavine-I, whereas expression of a modified version (B), comprising the N-terminal signal peptide from Pdi1, resulted in production of approximately half of this amount. Complete deletion of the N-terminal sequence (C) abolished the function of EasE_Aj, and essentially no chanoclavine-I was detected.

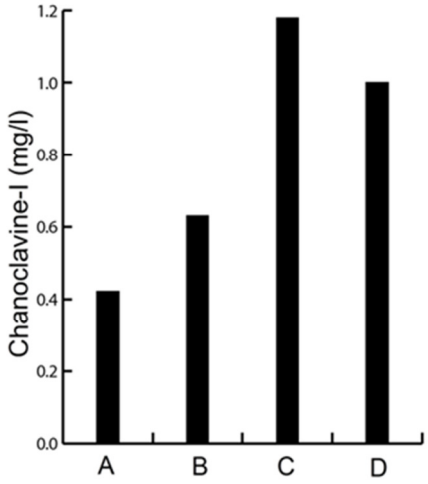

FIG 6 Concentration (mg/l) of chanoclavine-I, was measured in the growth medium, of a chanoclavine-I producing control strain (A) and after over-expression of Pdi1 (B), Ero1(C), or Fad1 (D). Over-expression of these native yeast genes all resulted in an increased production of chanoclavine-I, relative to the control. All strains expressed the integrated, heterologous pathway to chanoclavine-I.