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Redundant synthesis of a conidial polyketide by two distinct secondary metabolite clusters in *Aspergillus fumigatus*

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Summary

Filamentous fungi are renowned for the production of bioactive secondary metabolites. Typically, one distinct metabolite is generated from a specific secondary metabolite cluster. Here, we characterize the newly described tryptacidin (*tpc*) cluster in the opportunistic human pathogen *Aspergillus fumigatus*. We find that this cluster as well as the previously characterized endocrocin (*enc*) cluster both contribute to the production of the spore metabolite endocrocin. Whereas tryptacidin is eliminated when only *tpc* cluster genes are deleted, endocrocin production is only eliminated when both the *tpc* and *enc* non-reducing polyketide synthase-encoding genes, *tpcC* and *encA*, respectively, are deleted. EncC, an anthrone oxidase, converts the product released from EncA to endocrocin as a final product. In contrast, endocrocin synthesis by the *tpc* cluster likely results from incomplete catalysis by TpcK (a putative decarboxylase), as its deletion results in a nearly 10-fold increase in endocrocin production. We suggest endocrocin is likely a shunt product in all related non-reducing polyketide synthase clusters containing homologues of TpcK and TpcL (a putative anthrone oxidase), e.g. geodin and monodictyphenone. This finding represents an unusual example of two physically discrete secondary metabolite clusters generating the same natural product in one fungal species by distinct routes.

Introduction

Fungi are well known for the ability to synthesize bioactive secondary metabolites (SMs) of diverse chemical structure and complexity. The dominant SM-producing taxa belong to several genera of filamentous ascomycetes. Secondary metabolites are generally thought to contribute to the fitness of filamentous fungi, primarily as protection from abiotic stress (e.g.

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melanins) and/or for niche securement or defense in conflicts with other microbes or insects (e.g. toxins) (reviewed in Rohlfs and Churchill, 2011; Scherlach *et al.*, 2013). The same or similar metabolites can also serve as virulence factors in pathogenic fungi, including plant (Friesen *et al.*, 2008) and animal (Dagenais and Keller, 2009) pathogens.

Aspergillus fumigatus is the primary causative agent of invasive aspergillosis (IA) among immunocompromised individuals (Brakhage, 2005; Maschmeyer *et al.*, 2007). This species is notorious for producing a plethora of SMs (e.g. gliotoxin, helvolic acid, hexadecyloastochrome, tryptacidin, endocrocin, neosartoricin, and fumagillin) shown to exhibit immunomodulatory and/or cytotoxic properties that are thought to facilitate the pathogenicity of this fungus (Amitani *et al.*, 1995; Bok *et al.*, 2005; 2006; Spikes *et al.*, 2008; Lodeiro *et al.*, 2009; Gauthier *et al.*, 2012; Berthier *et al.*, 2013; Chooi *et al.*, 2013; Yin *et al.*, 2013), although their ecological role may relate to their antimicrobial activity (Carberry *et al.*, 2012; Kang *et al.*, 2013) and UV protective capacity (Allam and Abd El-Zaher, 2012).

Secondary metabolism is spatially and temporally regulated in fungi with metabolites produced in different compartments of the fungal thallus (Lim and Keller, 2014; Kistler and Broz, 2015). Mycelial SMs such as gliotoxin and helvolic acid have been extensively studied over the last decades (Amitani *et al.*, 1995; Bok *et al.*, 2006; Spikes *et al.*, 2008; Kwon-Chung and Sugui, 2009). However, with the exception of dihydroxynaphthalene (DHN) melanin (Jahn *et al.*, 1997; Sugui *et al.*, 2007; Bayry *et al.*, 2014), the spore-borne SMs are relatively understudied, despite spores being crucial to the fungus for dispersal and colonization of new substrates.

Recent studies have identified two spore metabolites that may contribute to the virulence of *A. fumigatus*. Endocrocin, a spore-borne product of a non-reducing polyketide synthase (NR-PKS) in *A. fumigatus*, was demonstrated to inhibit neutrophil migration both *in vitro* and *in vivo*, and deletion of the endocrocin polyketide synthase-encoding *encA* yielded a less pathogenic strain using the *Toll*-deficient *Drosophila* model (Berthier *et al.*, 2013). Another spore metabolite, tryptacidin, was shown to exhibit cytotoxic properties against both the A549 pulmonary adenocarcinoma cell line and human bronchial epithelial cells (Gauthier *et al.*, 2012). Interestingly, tryptacidin, first isolated from *A. fumigatus*, was identified as an anti-protozoal agent (Balan *et al.*, 1963; Turner, 1965), and the conidia of *A. fumigatus* have multiple reported defences against predation by soil amoebae (Van Waeyenbergh *et al.*, 2013; Hillmann *et al.*, 2015).

Despite being isolated more than 50 years ago, the genes responsible for tryptacidin production in *A. fumigatus* have not been identified. Tryptacidin is similar in structure to polyketides that belong to the clade of NR-PKSs involved in the synthesis of anthraquinone-derivatives, which include geodin in *Aspergillus terreus* (Nielsen *et al.*, 2013), monodictyphenone in *Aspergillus nidulans* (Chiang *et al.*, 2010; Sanchez *et al.*, 2011; Simpson, 2012) and endocrocin in *A. fumigatus* (Lim *et al.*, 2012). These NR-PKSs lack the canonical thioesterase (TE) domain responsible for releasing the nascent products from the enzyme. Instead, a separate gene encoding a metallo- β -lactamase type thioesterase (M β L-TE) is located adjacent to these NR-PKSs (Awakawa *et al.*, 2009). These two enzymes

catalyse the first steps in the biosynthesis of polyketides of this TE-less NR-PKS clade, discussed in recent reviews (Ahuja *et al.*, 2012; Chooi and Tang, 2012). Genome mining in *A. fumigatus* identified three NR-PKSs, two of which have been characterized and found to synthesize endocrocin (Lim *et al.*, 2012; Berthier *et al.*, 2013) and neosartoricin (Chooi *et al.*, 2013).

Here, we found that the third TE-less NR-PKS is responsible for the biosynthesis of the polyketide tryptacidin. The 13-gene tryptacidin (*tpc*) cluster is nearly identical to the 13-gene *ged* NR-PKS cluster, and several *tpc* genes also share homology with both the 12-gene *mdp* cluster and the 4-gene *enc* cluster. Unexpectedly, we found that both the *tpc* and *enc* clusters contribute to the production of endocrocin, with the former cluster ultimately producing tryptacidin. Through *tpc* and *enc* gene deletions coupled with metabolite profiling of these strains, we characterized two distinct routes to endocrocin production differentiated by early enzymatic steps in their respective pathways.

Results

Identification of the tryptacidin-producing gene cluster

The cluster containing the NR-PKS-encoding gene AFUA_4G14560 (hereafter referred to as *tpcC*) was first identified as one of the SM clusters regulated by the global regulator of secondary metabolism, LaeA (Perrin *et al.*, 2007). Analysis of the product template (PT) domain of TpcC shows that it belongs to the same clade of NR-PKS as the monodictyphenone PKS of *A. nidulans* (Chiang *et al.*, 2010), the endocrocin PKS of *A. fumigatus* (Lim *et al.*, 2012), and the recently described geodin PKS of *A. terreus* (Nielsen *et al.*, 2013) (Fig. 1A and Table S1). The PT domains of PKSs determine the regio-selectivity of cyclization during the synthesis of the polyketide backbone (Crawford *et al.*, 2009) and have been used for phylogenetic analyses. The phylogenetic group of PKSs to which TpcC belongs has previously been designated Group V1 (Li *et al.*, 2011), a subdivision of Group V (Li *et al.*, 2010) which, with a few exceptions, make a C6–C11 bond during the initial cyclization (Ahuja *et al.*, 2012).

The set of genes surrounding *tpcC* also has significant identity to the monodictyphenone, endocrocin, and geodin biosynthetic clusters (Fig. 1B). Genetic characterization of the geodin-producing cluster from *A. terreus* (Nielsen *et al.*, 2013) highlighted a high degree of similarity between these two clusters and their final products (Fig. 1B and Table S2). Given this similarity, we hypothesized that *tpcC* was the most likely candidate to initiate tryptacidin synthesis in *A. fumigatus*. While initial *in silico* predictions of the tryptacidin biosynthetic gene cluster spanned a 66 kb region with 31 genes (AFUA_4G14420 – AFUA_4G14730) (Khaldi *et al.*, 2010; Medema *et al.*, 2011; Inglis *et al.*, 2013), homology with the geodin cluster suggests the *tpc* cluster consists of a 25 kb region with 13 genes: AFUA_4G14580 through AFUA_4G14460, which we term *tpcA-M* (Fig. 1B and Table S2).

In order to confirm the assignment of these genes to tryptacidin biosynthesis, we created deletion mutants of *tpcC*, encoding the NR-PKS, and *tpcB*, encoding the M β L-TE (Table S2 and Fig. S1). Deletion of either gene resulted in loss of tryptacidin and pathway intermediates including questin as determined by comparison to purified standards (Fig. 2A–

C; Figs S2 and 3). Additionally, we identified two Zn₂Cys₆ transcription factor-encoding genes in the region of the *tpc* cluster, namely, *tpcE* and AFUA_4G14590 (Table S2). Deletion of *tpcE* but not AFUA_4G14590 resulted in loss of tryptacidin and questin (Fig. 2D–F; Fig. S4). TpcE is a homologue of AflR, the sterigmatocystin/aflatoxin Zn₂Cys₆ transcription factor (Woloshuk *et al.*, 1994). The co-activator AflS (formerly called AflJ) is also required for sterigmatocystin/aflatoxin synthesis (Woloshuk *et al.*, 1994; Ehrlich *et al.*, 2012) and enhances AflR activity. An *aflS* homologue, *tpcD*, is located next to *tpcE*, and its deletion also resulted in loss of tryptacidin (Fig. 2D–F, Fig. S4). We also confirmed regulation of the tryptacidin and endocrocin clusters by LaeA and the conidiation-specific transcription factor BrlA (Mah and Yu, 2006) (Fig. 2G–I, Fig. S5) as reported or suggested by earlier studies (Perrin *et al.*, 2007; Gauthier *et al.*, 2012). Both production of metabolites and gene expression showed BrlA to more tightly regulate the clusters than LaeA.

We present a likely biosynthetic pathway for tryptacidin synthesis (Fig. 3) based on these data and proposed and characterized polyketide biosynthetic pathways, primarily geodin, aflatoxin, monodictyphenone and prenyl xanthenes (Henry and Townsend, 2005a,b; Chiang *et al.*, 2010; Sanchez *et al.*, 2011; Simpson, 2012; Nielsen *et al.*, 2013).

Endocrocin produced by both the *tpc* and *enc* clusters through distinct routes

Because tryptacidin and endocrocin are both regulated by LaeA and BrlA (Fig. 2G – I) and the PKSs involved in their syntheses belong to the same phylogenetic clade, Group V (Li *et al.*, 2010), we thought it possible that their biosyntheses might have a unique interrelationship. Therefore, we assessed the AF293 strain, which produces both endocrocin and tryptacidin, to determine if any such relationship existed. In contrast to the *encA* mutation in the CEA10 background that displayed a complete loss of endocrocin (Lim *et al.*, 2012), we found that the AF293 *encA* mutant surprisingly still produced endocrocin, although at lower levels than wild type (WT; Fig. 4A – C). Extracts of the *tpcC* strain also appeared to show a reduction, but not elimination, of endocrocin relative to the wild-type control (Fig. 4A – C). Previous work had characterized the *enc* cluster in strains derived from the *A. fumigatus* isolate CEA10 (Lim *et al.*, 2012). However, this strain does not produce tryptacidin or its intermediates, despite the presence of the *tpc* cluster (Fig. S6). Examination of the nucleotide sequence of the *tpc* cluster in CEA10 uncovered a single-nucleotide insertion in the fourth exon of *tpcC*. This is predicted to result in a frameshift and premature termination codon, which would explain the inability of this isolate to produce these metabolites (Table S3).

Given this, as well as the similarity of the genes encoding the initial enzyme activities of the two clusters (Lim *et al.*, 2012), we hypothesized that the source of endocrocin in the AF293 *encA* mutant might be the *tpc* cluster (Fig. 4G). We therefore created several *encA*/ *tpcC* double deletion mutants and found that all of them exhibited a complete loss of endocrocin production (Fig. 4A – C). This observation confirms that the tryptacidin biosynthetic cluster contributes to the production of endocrocin and indicates that, in wild-type AF293, endocrocin is derived from both the *tpc* and *enc* clusters. Conversely, it does not appear that the *enc* cluster contributes to the tryptacidin pathway as demonstrated by complete loss of tryptacidin and its precursors in *tpc* cluster deletants despite the presence of an intact *enc*

cluster (Fig. 2A – F). This might suggest that the pathways are independent or perhaps compartmentalized within the cell.

Both trypacidin and endocrocin have been reported as spore metabolites. To more thoroughly assess tissue localization of endocrocin produced by either pathway, spore (Fig. 4D), conidiophore (containing fallen spores and some surface mycelium; Fig. 4E), and mycelia/agar (Fig. 4F) fractions were analysed for endocrocin in both the *encA* mutant and *tpcC* mutants. Supporting earlier studies, endocrocin was primarily localized to the spore fraction (Fig. 4D). Lesser amounts were found in conidiophore and mycelial fractions (Fig. 4E – F). Interestingly, a small amount of endocrocin was detectable in the mycelial fraction of the *encA* mutant but not of the *tpcC* mutant, suggesting more specific localization of the endocrocin produced by the *enc* cluster to the spore (Fig. 4F).

We asked whether the redundancy of endocrocin production could be attributed to utilization of an early intermediate common to both pathways by downstream enzymes in the endocrocin pathway. Specifically, we asked whether the monooxygenase EncC (the first decorating enzyme in the endocrocin biosynthetic pathway after release of the backbone from EncA by EncB; Fig. 4G) might use early pathway intermediates derived from either the *enc* or *tpc* clusters as a substrate to generate endocrocin. EncC possesses similarity to HypC, an anthrone oxidase involved in a similar conversion in aflatoxin biosynthesis (Ehrlich *et al.*, 2010), and deletion of EncC results in loss of endocrocin in the CEA10 background (Lim *et al.*, 2012). If EncC could utilize precursors from early steps in trypacidin synthesis, we hypothesized that deletion of *encC* would result in complete loss of endocrocin, much like the *encA*/ *tpcC* mutants. However, endocrocin production in the AF293 *encC* mutant was similar to that of the AF293 *encA* mutant showing a reduction but not elimination of endocrocin (Fig. 4A – C). While these results cannot rule out the possibility of intermediate sharing between the two pathways, they are consistent with the hypothesis that endocrocin is produced as a shunt product of the trypacidin pathway.

The hypothetical endocrocin shunt would likely occur from the early steps in the trypacidin pathway. Previous work had shown that deletion of *mdpH* in *A. nidulans* resulted in accumulation of endocrocin (Chiang *et al.*, 2010), and *tpcK* and *tpcL* are homologous to the 5' and 3' halves of *mdpH* respectively (Fig. 1B and Fig. 5A). TpcL also shows homology to the anthrone oxidases HypC and EncC, whereas TpcK is a putative decarboxylase, based on the prediction of the same activity for MdpH (Chiang *et al.*, 2010). We therefore made single and double deletions of *tpcK* and *tpcL* to address if either of the encoding enzyme activities contributed to endocrocin production.

Deletion of *tpcK* increased endocrocin production nearly 10-fold and exhibited concomitant loss of downstream trypacidin pathway metabolites, emodin and questin (Fig. 5B–D; Fig. S7). This observation is consistent with the proposed role of TpcK as a decarboxylase (Figs 2G and 4G) and similar to the result of *mdpH* deletion in *A. nidulans*. Deletion of *tpcL* resulted in modest decrease in the production of questin but an approximately twofold increase in endocrocin and emodin (Fig. 5B–D; Fig. S7). This suggests that the proposed enzymatic step for TpcL (Figs 2G and 4G) might be catalysed by other oxidases in the genome or may occur spontaneously. Double deletion of *tpcK* and *tpcL* resulted in a similar

phenotype to the *tpcK* mutant, suggesting that *tpcK* is upstream of *tpcL* in the tryptacidin pathway. A third putative anthrone oxidase-encoding gene, AFUA_4G09250, was found in the *A. fumigatus* genome, however, its deletion had no effect on the levels of metabolites in the endocrocin or tryptacidin pathways (Fig. 5B–D; Fig. S7).

We also examined the metabolic output of deletions of *tpcK*, *tpcL*, *tpcK/L*, or AFUA_4G09250 in a *encC* background. These mutants yielded approximately half the amount of endocrocin relative to the amount produced by their respective single deletants in a wild-type *encC* background, but no other significant changes (Fig. 5E – G). Thus, it appears that endocrocin production from the tryptacidin pathway results from incomplete conversion of atrochrysone carboxylic acid to downstream products, specifically by TpcK. Taken together, these results strongly support independent mechanisms leading to endocrocin biosynthesis by the *tpc* and *enc* clusters where one cluster yields endocrocin as a shunt metabolite (*tpc*) and one as an end metabolite (*enc*) (Fig. 4G).

Contribution of *tpc* and *enc* clusters to virulence in *Toll*-deficient *Drosophila melanogaster*

An earlier study had shown that *encA* deletion resulted in a less virulent strain using the *Toll*-deficient *Drosophila* model of IA (Lionakis *et al.*, 2005; Berthier *et al.*, 2013). We again used this model to compare the pathogenicity of both single *encA* and *tpcC* deletion mutants and the double deletant to wild type (Fig. S8). The results show that the *encA* mutant has significantly decreased virulence in two of three trials, while the *tpcC* mutant has no consistent effect. The *encA/tpcC* mutant is also significantly impaired in virulence in two of the three trials.

Discussion

The *tpc* cluster constitutes the fifth described example of a LaeA- and BrIA-regulated spore SM cluster alongside DHN melanin, fumigaclavines, endocrocin, and the fumiquinazolines (Coyle *et al.*, 2007; Perrin *et al.*, 2007; Twumasi-Boateng *et al.*, 2009; Lim *et al.*, 2012; 2014; Upadhyay *et al.*, 2013). Although tryptacidin was initially reported to be isolated from the mycelium (Turner, 1965), it was later found to localize to the conidia (Parker and Jenner, 1968; Gauthier *et al.*, 2012), a finding supported by our work. In an unexpected twist, we find that both the *tpc* and *enc* clusters contribute to the synthesis of endocrocin. To our knowledge, only two prior studies have described two physically discrete clusters contributing to the synthesis of the same metabolite in one fungal species (Forseth *et al.*, 2013; Guo *et al.*, 2015). This finding is in contrast to the recently discovered phenomenon of superclusters, in which a single co-regulated cluster can produce two distinct metabolites (e.g. fumagillin and pseurotin) (Wiemann *et al.*, 2013), and thus expands our concept of SM plasticity in filamentous fungi.

The *tpc* and *enc* clusters, along with the *ged* and *mdp* clusters, contain a TE-less NR-PKS coupled with a M β L-TE, characteristic of this phylogenetic clade of PKSs (Fig. 1A). In three of these four clusters, these genes are present as a divergently oriented pair (Fig. 1B). In addition to maintaining this gene pair, the early biosynthetic steps proposed for all four metabolites are very similar, consisting of the PKS-M β L step followed by anthrone oxidase activity (Figs 2G and 4G) (Chiang *et al.*, 2010; Lim *et al.*, 2012; Nielsen *et al.*, 2013).

Interestingly, *in vitro* expression of the geodin PKS-encoding gene, *gedC* (formerly called ACAS), and M β L-TE-encoding gene, *gedB* (formerly called ACTE), yielded endocrocin, emodin, and their precursors (Awakawa *et al.*, 2009). Also, as mentioned, deletion of *mdpH* of the monodictyphenone cluster resulted in accumulation of endocrocin (Chiang *et al.*, 2010). Coupling these data with our finding that the *tpc* cluster can produce endocrocin, likely because of incomplete TpcK activity, we suggest that endocrocin production may be generated as a shunt product from NR-PKS/ M β L-TE clusters containing TpcK and TpcL homologues or produced directly by those clusters containing a TpcL (but not TpcK) homologue. The anthrone oxidase and decarboxylase activities are keys in endocrocin metabolism, either directing to (TpcL/EncC) or diverting from (TpcK/MdpH) its synthesis.

Previous studies (Awakawa *et al.*, 2009) suggest that the NR-PKS/M β L-TE pairing alone could produce some amount of endocrocin which may be oxidized non-enzymatically from an anthrone precursor (Fig. 4G). Deletion of the anthrone oxidase HypC in *Aspergillus parasiticus* revealed a similar situation where conversion of norsolorinic acid anthrone to norsolorinic acid also occurred non-enzymatically (Ehrlich *et al.*, 2010). Deletion of *tpcL* in our study still allowed for emodin accumulation, presumably through processing of emodin anthrone either non-enzymatically or by another oxidase encoded in the genome.

Interestingly, endocrocin production is reported not only from numerous fungi (Kurobane *et al.*, 1979; Räsänen *et al.*, 2000; Yang *et al.*, 2013), but also insects (Kikuchi *et al.*, 2011) and plants (Jan *et al.*, 2015) which may reflect yet other biosynthetic pathways leading to endocrocin.

From where might the additional genes in the more complex NR-PKS/M β L-TE clusters derive? The *tpc* cluster was referenced in a 2007 study of the evolution of the aflatoxin gene cluster (Carbone *et al.*, 2007). Aflatoxin is a polyketide generated from a large NR-PKS cluster containing several genes with significant similarity to *tpc* cluster genes, in addition to the already noted *hypC*, which was not yet discovered in 2007. In this paper, the authors identify modules, consisting primarily of highly correlated gene pairs that they propose are duplicated and evolved in secondary metabolite gene clusters. At the time, five genes in what we now know as the *tpc* cluster were speculated as having arisen from a hypothetical cluster ancestral to the aflatoxin cluster. These were *tpcD/E*, which are homologous to *aflR/S*; *tpcG/I*, which are homologous to *aflX/Y*; and *tpcC*, homologous to *aflC*. Expansion of an ancestral *enc*-like cluster can be envisioned by ‘capture’ of such modules. It is possible that the current *enc* and *tpc* clusters both arose from a small ancestral cluster in *A. fumigatus*. Alternatively, the *enc* cluster, comprising fewer genes than the *tpc* cluster, may have arisen from loss of modules from an ancestral *tpc*-like cluster.

Though previous predictions of the tryptacidin cluster range from AFUA_4G14420- AFUA_4G14730 (Khaldi *et al.*, 2010; Medema *et al.*, 2011; Inglis *et al.*, 2013), the cluster is nearly identical to the geodin-producing cluster in *A. terreus* (Nielsen *et al.*, 2013). Twelve of the 13 genes in these clusters are homologous, with only the methyltransferase-encoding *tpcH* and halogenase-encoding *gedL* unique to each cluster. These two activities are reflected in the chemical structures of their respective end metabolites (Fig. 1B). Interestingly, these unique genes are on either side of a five-gene inversion, suggesting a possible mechanism for introduction and maintenance of variation in an ancestral SM-

producing gene cluster. Variations of fungal SM clusters resulting from inversions and deletions are common and contribute to the production of biosynthetically related but different end metabolites, e.g. the aflatoxin and sterigmatocystin gene clusters of *A. flavus* and *A. nidulans* (Hodges *et al.*, 2000; Lee *et al.*, 2006; Carbone *et al.*, 2007) and the sirodesmin and gliotoxin clusters of *Leptosphaeria maculans* and *A. fumigatus* (Gardiner *et al.*, 2004; Fox and Howlett, 2008).

The *enc* and *tpc* clusters are both regulated by LaeA and BrlA, while the *tpc* cluster is further regulated by the TpcD/E pair. The acquisition or maintenance of *tpcD/E* might allow for more dynamic production of tryptacidin and its intermediates, many of which are bioactive (Fujimoto *et al.*, 1999; Ohashi *et al.*, 1999; Choi *et al.*, 2007; Shrimali *et al.*, 2013). The presence of cluster-specific transcription factors potentiates a more nuanced activation of the cluster perhaps to time the production of the metabolites. As suggested for aflatoxin biosynthesis (Meyers *et al.*, 1998; Du *et al.*, 2007; Roze *et al.*, 2007), and consistent with observations of the geodin cluster (Nielsen *et al.*, 2013), the AflR/S homologues may enable the uncoupling of early and late phases of the biosynthetic pathway. In total, the coordinated timing of this and the conidiation-specific level of regulation, involving BrlA, may facilitate the specific accumulation of the end metabolite, tryptacidin, into the spore.

For this study, we utilized *A. fumigatus* strain AF293, which produces tryptacidin and endocrocin, however, the previous study characterizing the *enc* cluster used CEA10-derived strains, which do not produce tryptacidin. There are other examples of differential production of a metabolite between strains of a given species, including *A. fumigatus* (Frisvad *et al.*, 2009). In some cases, genetic defects responsible for this differential metabolite production have been described in the cluster. For example, in AF293, a point mutation in *ftmD* prevents the strain from producing fumitremorgins in contrast to CEA10 and *A. fumigatus* strain BM939 (Kato *et al.*, 2013). Further examples have been suggested in *Aspergillus niger* (Andersen *et al.*, 2011), and such defects are thought to have resulted in the loss of aflatoxin production in some *Aspergillus oryzae* strains (Lee *et al.*, 2006; Kiyota *et al.*, 2011).

In summary, the newly described tryptacidin cluster and the endocrocin cluster both produce endocrocin through distinct routes in *A. fumigatus*. The tryptacidin and endocrocin clusters are both regulated by LaeA and BrlA, and their end metabolites are localized to the asexual spores. Relatively little is known about the biological significance of endocrocin and tryptacidin synthesis, but we speculate that they may have a role in protection of the spore from abiotic stressors. Endocrocin and precursors of tryptacidin (e.g. questin and emodin) are pigmented anthraquinones. Anthraquinones have been shown to provide protection from UV radiation to the producing organism (Nybakken *et al.*, 2004) and may contribute to protection to *A. fumigatus* as well. Other than the trypanocidal activity of tryptacidin (Balan *et al.*, 1963), additional studies are required to confirm any biological function of these metabolites and the redundancy in endocrocin production for the fungus.

Experimental procedures

Fungal strains and growth conditions

All strains used in this study are listed in Table S4. Mutants were derived from *pyrG1* or *argB1/pyrG1* auxotrophic backgrounds of *Aspergillus fumigatus* strain AF293 (Oshero et al., 2001; Xue et al., 2004). Stocks of each strain were stored in 30% (v/v) glycerol in 0.01% (v/v) TWEEN-80 at -80°C . Strains were activated and grown on solid glucose minimal medium (GMM) (Shimizu and Keller, 2001) with appropriate supplements at 37°C for 3 days for spore collection. For *pyrG1* auxotrophs, 5.2 mM uridine and 5 mM uracil were added as supplements, and for *argB1* auxotrophs, 5.7 mM L-arginine was added as a supplement. Spores were collected in 0.01% (v/v) TWEEN-80 and enumerated using a hemacytometer. For isolation of genomic DNA for polymerase chain reaction (PCR) and Southern blot, 10 ml of liquid minimal medium (Shimizu and Keller, 2001) with yeast extract was inoculated with spores from solid medium and grown overnight at 37°C . For assessment of metabolite production, 5 μL of a suspension of 2×10^6 spores ml^{-1} of each strain were point inoculated on solid GMM and grown at 29°C for 120 h without light selection. For tissue-specific metabolite analysis, this time was increased to 192 h. The conidial fraction of the culture was collected by tapping the plate with a spatula with the lid down, the conidiophore/surface mycelial fraction (also containing residual spores) was collected by scraping into 5 ml 0.01% (v/v) TWEEN-80, and the mycelial fraction was collected by taking three 1.5 cm diameter cores after rinsing the plate with ddH_2O .

Construction of mutants

The mutants used in this study were created using the double-joint PCR method (Szewczyk et al., 2006). Genomic DNA was isolated as previously described (Shimizu and Keller, 2001). Generation of protoplasts and transformation were performed as previously described (Szewczyk et al., 2006). Primers (Table S5) were designed to amplify 800–1000 bp flanks with 20 bp of overlap with the selection cassette-containing plasmid (Table S6) with DNASTAR's LASERGENE12 suite (DNASTAR; Madison, WI) and PRIMER3 (Koressaar and Remm, 2007; Untergasser et al., 2012). For deletion strains, the plasmid used was pJW24 (Calvo et al., 2004), encoding the *A. parasiticus pyrG* gene, or pJMP4 (Sekonyela et al., 2013), encoding the *A. fumigatus argB* gene. The *A. fumigatus akuA* gene was deleted in AF293.6 (Xue et al., 2004) using *A. parasiticus pyrG* amplified from pJW24 (Calvo et al., 2004) to produce TFYL43.2. To generate TFYL44.1, *A. parasiticus pyrG* was removed from the *akuA* locus by transforming strain TFYL43.2 with single-joint PCR product containing 0.5–1 kb genomic DNA fragments flanking the *pyrG* sequence. TFYL45.1 was generated from TFYL43.2 by replacing the *A. parasiticus pyrG* at the *akuA* locus with firefly luciferase constitutively expressed by the *A. nidulans gpdA* promoter amplified from pJMP147 (J. Palmer and N. Keller, unpubl.). For both TFYL44.1 and TFYL45.1, transformants were plated onto sorbitol minimal media supplemented with the above-mentioned concentrations of uridine, uracil, arginine and sub-inhibitory concentration (0.75 mg ml^{-1}) of 5-fluoroorotic acid (5-FOA). Both *encA* and *encC* were deleted in strain TFYL45.1 using *A. parasiticus pyrG* amplified from pJW24 to generate strains TFYL68.1 and TFYL69.1 respectively. *tpcC* was deleted in strain TFYL45.1 using *A. fumigatus argB* amplified from pJMP4 to generate strain TFYL71.1. To generate the double *encA* and *tpcC* deletion mutant (TFYL72.1), *tpcC*

was deleted from strain TFYL68.1 using *A. fumigatus argB* amplified from pJMP4 (Sekonyela *et al.*, 2013). Both TFYL68.1 and TFYL69.1, arginine auxotrophs, were transformed with pJMP4 to generate the prototrophs TFYL73.1 and TFYL74.1, respectively, while TFYL71.1, a uridine and uracil auxotroph, was transformed with pKJA12.1 (K. Affeldt and N. Keller, unpubl.) to generate the prototroph TFYL76.1. Transformants were screened for proper integration of the construct first by PCR, then by Southern blot (Fig. S1). For all auxotrophic mutants, maintenance of the mutant allele(s) was confirmed via PCR after complementation to prototrophy. The wild-type control used for all strains created in the *akuA* background, TFYL81.1, was created from TFYL43.2 by complementation to prototrophy with *A. fumigatus argB*. This strain was comparable to AF293 in production of the metabolites assessed in this study.

Comparative genomic analysis

Sequence information and genome annotation were derived from the *Aspergillus* Genome Database aspgd.org (Cerqueira *et al.*, 2014). Comparison of orthologous genes in various fungal species was carried out using the SYBIL browser hosted at AspGD. The National Center for Biotechnology Information's BLAST (Altschul *et al.*, 1990) was used to manually supplement the annotations of AspGD and the predictions made by the SYBIL browser. DNASTAR's LASERGENE 12 suite (including SEQUENCER, SEQUENCER Pro and EDITOR) was used for sequence analysis (DNASTAR, Madison, WI). For phylogenetic tree construction CLUSTAL OMEGA (Sievers *et al.*, 2011) was used to align a large set of NR-PKSs (Table S1), and UGENE was used to extract the PT domain based on the canonical PT domain from the *A. parasiticus* PksA. These PT domains were then realigned and used as the basis for the phylogenetic tree with CLUSTAL OMEGA and FIGTREE (Rambaut, 2007).

SM extraction and chromatography

For tissue-specific metabolite extraction from the conidial fraction (see above), the spores were collected in 6 ml of 50:50 ethyl acetate : methanol, the conidiophore fraction was extracted with an equal volume (5 ml) of ethyl acetate. The mycelial fraction and all other samples were processed as follows: A 1.5 cm core was taken from each plate grown as described above, and homogenized in 2.5 ml of double-distilled water (ddH₂O) in a glass extraction vial with a Kinematica homogenizer. To each vial, 2.5 ml of ethyl acetate was added, vortexed and allowed to steep overnight. The vials were then centrifuged at 1100 g for 3 min and 1.9 ml of the organic layer was transferred to a new vial. This extract was allowed to evaporate at room temperature. For thin-layer chromatography (TLC), the extracts were dissolved in 50 µL ethyl acetate and 10 µL was spotted to a 250 µm silica gel plate (Whatman, Cat#15-4410-222), which was then placed in a mobile phase of toluene, ethyl acetate and formic acid in a 50:40:7 volume ratio. The plate was then imaged at 366 and 254 nm using a FOTO/Analyst Investigator gel imaging system (Fotodyne). For high performance liquid chromatography (HPLC) analysis, the extracts were re-suspended in 50% (v/v) acetonitrile in ddH₂O and filtered using a 0.2 µm syringe filter. The samples were analysed using a Perkin Elmer Flexar Instrument as previously described (Lim *et al.*, 2014), but with 1% (v/v) formic acid in solvents A (ddH₂O) and B (acetonitrile), a 1.5 ml min⁻¹ flow rate, and using the following modified program: equilibration in 20% B for 10 min before each sample injection, 20% B for 2 min, ramp to 50% B over 20 min, ramp to 100%

B over 1 min, hold at 100% B for 5 min. Strains were analysed in duplicate, the peak areas for each compound averaged and calculated as a proportion of their respective wild-type controls, either AF293 or TFYL81.1 (Table S4). TFYL81.1 was comparable to AF293 in production of the metabolites assessed in this study. Statistical significance of pairwise comparisons between the metabolite profiles of different strains was assessed by multiple *t*-tests with a threshold of 0.05 and the Holm-Sidak correction for multiple comparisons in Prism (GRAPHPAD Software). Trypacidin standard was obtained from Dr. Olivier Puel (UMR1331 ToxAlim, French National Institute for Agricultural Research, INP, UPS, Toulouse, France), and endocrocin, emodin and questin standards from Dr. Clay Wang (USC School of Pharmacy, 1985 Zonal Avenue, Los Angeles, California, U.S.A.) (Fig. S2).

Drosophila assays

Flies were generated by crossing a *Drosophila melanogaster* line carrying a thermosensitive allele of *Toll* (Tl r632) with a line carrying a null allele of *Toll* (Tl I-RXA) (Lionakis *et al.*, 2005). Five to seven-day-old adult female *Toll*-deficient flies were used for all experiments. Twenty to thirty-two flies were infected with each *A. fumigatus* strain. *A. fumigatus* isolates were grown on yeast extract agar glucose at 25°C. Conidia were collected from 2-day-old cultures in sterile 0.9% (w/v) saline, enumerated using a hemacytometer and adjusted to a concentration of 5×10^7 conidia ml⁻¹. The dorsal thorax of CO₂ anaesthetized flies was punctured using a sterile 10 µm needle dipped in this conidial suspension. As a negative control, flies were punctured with a needle that was not dipped in conidial suspension. Flies were monitored daily for survival over 7 days. Death within 3 h was considered a result of the injection procedure, and these flies were not included in subsequent analysis. The flies were housed in a 29°C incubator to maximize expression of the Tl r632 phenotype (Lionakis *et al.*, 2005). The *Toll*-deficient flies were transferred into fresh vials every 3 days. Each experiment was repeated three times on different days at a consistent time of day to eliminate variability due to circadian rhythm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A. Phylogenetic tree showing the relationships between the Group V PKSs in the aspergilli based on their PT domains. This is an excerpt of the tree described in the methods, chosen to include the Group V PKSs, with the orsellinic acid PKS, OrsA, as an outgroup. The tree shows that these PKSs fall into two main subgroups, Group V1 and V2 (Li *et al.*, 2011), with EncA basal to both. The PKSs corresponding to clusters shown in Fig. 1B are in bold.

B. The trypacidin gene cluster and its homologous characterized Group V1 NR-PKS gene clusters in the aspergilli. The producing species are listed above each cluster and their respective products are displayed below. All but the endocrocin cluster also have homologues of the paired transcriptional regulators of the aflatoxin gene cluster, AflR/AflS, shown in white. PKS genes are shown in gray, genes conserved in all clusters shown with a gray outline, and all other genes in black. Shaded regions connect homologous genes, and non-contiguous segments are separated by slashes.

Fig. 2. Representative chromatograms from HPLC analysis of wild-type (AF293) and trypacidin cluster mutants (*tpcB* and *tpcC*, A–C; *tpcE*, *tpcD*, and AFUA_4G14590 (590), D–F; *laeA* and *brlA*, G–I) at 285 nm (A, D and G) and 441 nm (B, E and H). These data are trimmed to show only the relevant ranges of retention times for detection of trypacidin (*tpc*), its precursors questin and emodin (qst and emd, respectively), and endocrocin (enc). Quantification of duplicate analyses is presented in the bar graphs (C, F and I), and asterisks represent statistical significance of the indicated pairwise comparisons with $\alpha = 0.05$.

Fig. 3.

The proposed biosynthetic pathway of tryptacidin. The protein putatively catalysing each step is indicated over the arrow, and the dehydration potentially occurs spontaneously. Genes-encoding enzymes in bold were deleted in this study, whereas the assignment of roles for enzymes that are in regular font and parentheses were made based on comparison to similar characterized biosynthetic pathways. The assignment of the methyltransferases TpcA, TpcH and TpcM was determined by analysis of a *tpcA* strain (Fig. S3) and comparison to the proposed cluster and biosynthetic pathway for geodin (Nielsen *et al.*, 2013). Note the conversion of questin to desmethylsulochrin, and similar reactions in monodictyphenone and aflatoxin biosynthesis, have multiple proposed mechanisms (Henry and Townsend, 2005a,b; Simpson, 2012).

Fig. 4.

Representative chromatograms from HPLC analysis of WT (TFYL81.1), *encA*, *encC*, *tpcC*, and *encA/tpcC* mutants at 285 nm (A) and 441 nm (B). These data are trimmed to show only the relevant ranges of retention times for detection of tryptacidin (tpc), its precursors questin and emodin (qst and emd, respectively), and endocrocin (enc). Quantification of duplicate analyses is presented in the bar graph (C). Quantification of triplicate analyses of the tissue-specific production and localization of endocrocin (detected at 441 nm) to the conidia (D), conidiophores (E) and mycelia/media (F) assessed by HPLC analysis of WT (TFYL81.1), *encA*, and *tpcC* mutants. Asterisks (C–F) represent statistical significance of the indicated pairwise comparisons with $\alpha = 0.05$. Comparison of

the early steps of the predicted trypacidin and endocrocin biosynthetic pathways (D). The protein putatively catalysing each step is indicated over the arrow, and the dehydration potentially occurs spontaneously.

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Fig. 5.

A diagram of the homologues of *tpcK* and *tpcL* in *A. fumigatus*, *A. terreus* and *A. nidulans* (A). Representative chromatograms from HPLC analysis of WT (TFYL81.1) and endocrocin and tryptacidin cluster mutants (*encC*, *tpcK*, *tpcL*, *tpcK/tpcL*, and AFUA_4G09250 (*250*), B–D; *encC*, *tpcK/encC*, *tpcL/encC*, *tpcK/tpcL/encC*, and AFUA_4G09250/ *encC* (*250/encC*), E–G) at 285 nm (B and E) and 441 nm (C and F). These data are trimmed to show only the relevant ranges of retention times for detection of tryptacidin (*tpc*), its precursors questin and emodin (*qst* and *emd*, respectively), and endocrocin (*enc*). Quantification of duplicate analyses is presented in the

bar graphs (D and G), and asterisks represent statistical significance of the indicated pairwise comparisons with $\alpha = 0.05$.

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