Prototype of an intertwined secondary-metabolite supercluster

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The hallmark trait of fungal secondary-metabolite gene clusters is well established, consisting of contiguous enzymatic and often regulatory gene(s) devoted to the production of a metabolite of a specific chemical class. Unexpectedly, we have found a deviation from this motif in a subtelomeric region of *Aspergillus fumigatus*. This region, under the control of the master regulator of secondary metabolism, LaeA, contains, in its entirety, the genetic machinery for three natural products (fumitremorgin, fumagillin, and pseurotin), where genes for fumagillin and pseurotin are physically intertwined in a single supercluster. Deletions of 29 adjoining genes revealed that fumagillin and pseurotin are coregulated by the supercluster-embedded regulatory gene with biosynthetic genes belonging to one of the two metabolic pathways in a noncontiguous manner. Comparative genomics indicates the fumagillin/pseurotin supercluster is maintained in a rapidly evolving region of diverse fungal genomes. This blended design confounds predictions from established secondary-metabolite cluster search algorithms and provides an expanded view of natural product evolution.

gene regulation | $Zn(II)_2Cys_6$ transcription factor | FapR | biosynthesis | cluster evolution

Filamentous fungi are well known for their ability to produce a variety of natural products, so-called secondary metabolites that are not essential for growth under laboratory conditions (reviewed in refs. 1 and 2). However, the maintenance of the genetic information allowing fungi to produce secondary metabolites suggests that these small molecules provide essential benefits in environmental niches ranging from protection from fungivory (reviewed in ref. 3) to chemical shields from UV radiation (4). Apart from providing evolutionary fitness to the producing organism in their natural habitat, many secondary metabolites are of major importance to humans because of their beneficial and deleterious effects as drugs and toxins, respectively.

Fungal secondary metabolism has been characterized by physical linkage of the genes required for synthesis of specific metabolites and the distinct enzymatic machinery encoded by these genes (1, 2, 5). For instance, most fungal secondary metabolites belong to one of four chemical classes that are characterized based on the key or backbone enzymes that consist of polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs), terpene cyclases (TCs), and prenyltransferases (PTs). Typically, cluster genes adjacent to these backbone genes code for accessory enzymes involved in either modification of the chemical scaffold, transcriptional control of cluster genes, transport of substrates and/or products, and resistance mechanisms. The most common regulatory genes of clusters encode fungal-specific C6 zinc binuclear cluster ($Zn(II)_2Cys_6$) transcription factors (6), which, in general, exert positive transcriptional regulation of most of the genes within a single cluster (7). In addition to cluster-specific transcription factors, a higher order of regulation was discovered in the global regulator of fungal secondary metabolism, LaeA, initially characterized in the genus *Aspergillus* (8–11) but later in other fungal genera (12–16). LaeA regulation of multiple secondary metabolite clusters in each species may or may not involve activation of cluster-specific $Zn(II)_2Cys_6$ factors (17–19).

Analysis of both LaeA and pathway-specific $Zn(II)_2Cys_6$ mutants have been useful in demarcating the ends of simple secondary-metabolite clusters (20, 21). However, this task is much greater for complex and lengthy clusters despite the existence of secondary-metabolite cluster search algorithms, such as SMURF and antiSMASH, that assign contiguous genes to production of a distinct secondary metabolite based on current knowledge of fungal clusters (22, 23). Here, using previously published microarray data of a Δ*laeA* mutant in *Aspergillus fumigatus* (24), we uncover the blending and regulation of a "supercluster" in the subtelomeric region of chromosome VIII (25–27). Our data presented here demonstrate an example of one fungal secondary-metabolite gene cluster in which the genes responsible for two pathways (pseurotin and fumagillin) are physically intertwined in the genomic context. These data correct the hallmark motif of two separated clusters predicted for pseurotin and fumagillin by current algorithms. Syntenic comparison of this region to *Neosartorya fischeri*, *Aspergillus clavatus*, and the entomopathogenic fungus *Metarhizium anisopliae* suggest a rapidly evolving "supercluster motif" in diverse fungal genomes.

Results

A Telomeric Region Containing Multiple Secondary Metabolite Key Enzymes on Chromosome VIII Is Positively Regulated by LaeA. Using microarray comparisons, Perrin et al. (24) identified a LaeA-regulated supercluster on the left arm of chromosome VIII

Significance

Filamentous fungi are well known for their ability to produce a wide range of natural products. Until now, biosynthetic genes contiguously aligned in a cluster have been associated with the production of a distinct natural product. This work demonstrates an additional layer of complexity in fungal secondary-metabolite gene clusters. In contrast to the view of stand-alone secondary-metabolite clusters, our collective data have revealed the existence of superclusters with intertwined biosynthetic genes involved in formation of more than one chemical product. Comparative genomics indicates this supercluster is maintained in a rapidly evolving region of diverse fungal genomes. This intertwined design confounds predictions from established secondary-metabolite cluster search algorithms and provides an expanded view of natural product evolution.

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(AfuA_8g00100-00720) (Fig. 1). More recently, this supercluster has been preliminarily characterized to encode for at least three bioactive metabolites, fumitremorgin, pseurotin, and fumagillin. Whereas the fumitremorgin gene cluster is well-characterized [AfuA_8g00170-00250 (28)], only one of the genes has been characterized for the pseurotin cluster [the PKS-NRPS hybrid-encoding gene *psoA*/*NRPS14*/AfuA_8g00540 (26)] and only three of the genes of the fumagillin cluster [TC-, PKS-, and acyltransferase (AT)-encoding genes *fma*-TC/*fmaA*/AfuA_8g00520, *fma*-PKS/*fmaB*/AfuA_8g00370, and *fma*-AT/*fmaC*/AfuA_8g00380 (27)] (Fig. 1). The cluster ends have not been delineated for any of these clusters but have been predicted based on microarray data (24) and computer models (22, 23).

The LaeA-Regulated Zn(II)$_2$Cys$_6$ Transcription Factor FapR Regulates Pseurotin and Fumagillin but Not Fumitremorgin Cluster Genes. The 170-kb region of chromosome VIII controlled by LaeA contains 69 putative ORFs (ref. 29 and Table S1). Of these 69 ORFs, only 1 (AfuA_8g00420/AfuB_086150) showed homology to proteins involved in transcriptional regulation. The identified protein has similarity to fungal-specific Zn(II)$_2$Cys$_6$ transcription factors, the proteins commonly associated with regulation of secondary-metabolite clusters in fungi (7). Regulation of Zn(II)$_2$Cys$_6$ proteins by LaeA has been associated with cluster regulation, such as LaeA regulation of the aflatoxin/sterigmatocystin regulator AflR (17).

We, therefore, thought it possible that LaeA regulation of all three secondary-metabolite clusters (fumagillin, pseurotin, and fumitremorgin) could be mediated by AfuB_086150 (Zn(II)$_2$Cys$_6$ transcription factor) and, thus, constructed a deletion mutant of AfuB_086150. Expression analysis of selected genes from each cluster indicated that LaeA regulated genes from all three clusters, whereas the transcription factor AfuB_086150 regulated 21 genes within the fumagillin and pseurotin gene clusters (Fig. 2). No significant difference between expression levels of the fumitremorgin NRPS, *ftmA*, was observed between the wild type and the ΔAfuB_086150 mutant (Fig. 2). In accordance with the transcriptional data, chemical data by HPLC–tandem MS (HPLC-MS/MS) demonstrate loss of fumagillin and pseurotin but no alteration in fumitremorgin production in the ΔAfuB_086150 mutant compared with the wild type (Fig. S1). Moreover, deletion of the putative cluster border genes (AfuB_086280-AfuB_086210 and AfuB_085980; Fig. 1 and Table S2) did not affect fumitremorgin, fumagillin, or pseurotin gene expression and concomitant biosynthesis (Fig. 2, Fig. S2, and Table 1). Considering the requirement of the putative transcription factor for expression of genes in both fumagillin and pseurotin clusters and concomitant product formation, we named AfuB_086150 FapR (fumagillin and pseurotin regulatory gene).

Characterization of FapR-Regulated Genes Reveals That Fumagillin and Pseurotin Biosynthetic Genes Are Physically Intertwined. To delineate which of the FapR-regulated genes participate in fumagillin and pseurotin biosynthesis, respectively, we generated single mutants for each putative cluster gene (Table S2 and Fig. 1). Unexpectedly, as detailed below, we found that two genes embedded in the fumagillin cluster and proposed to be involved in fumagillin synthesis were actually required for pseurotin synthesis.

Fumagillin Cluster. Confirming Lin et al. (27), single deletions of *fmaB* and *fmaC* resulted in production of the intermediate fumagillol (*m/z* 283) instead of fumagillin (Fig. S2, Fig. S3, and Table 1). Similarly, the *fmaA* deletion mutant lacked fumagillin and fumagillol production (Table 1, Fig. S2, and Fig. S3). We found four additional genes were required for fumagillin biosynthesis. Mutants lacking the putative *O*-methyltransferase–encoding gene (*fmaD*) produced demethyl-fumagillin (calculated for $C_{25}H_{33}O_7[M + H]^+$, *m/z* 445.2221; found, *m/z* 445.2218) and deletion of the putative phytanoyl-CoA-dioxygenase–encoding gene (*fmaF*) resulted in formation of demethyl-fumagillin-aldehyde (calculated for $C_{25}H_{33}O_6$ $[M + H]^+$, *m/z* 429.2272; found, *m/z* 429.2280) (Fig. S2, Fig S3, and Table 1). Deletion of the putative P450-encoding gene (*fmaG*) or the hypothetical-protein-encoding gene (*fmaE*) resulted in loss of fumagillin and fumagillol production. However, we did not observe accumulation of any putative intermediate or shunt product in these mutants. None of the other single deletion mutants between *fmaA* and *fmaB* affected fumagillin production (Table 1 and Fig. S2).

Pseurotin Cluster. We next looked at the genes previously assigned to pseurotin synthesis based on deduced protein functions (26) and on our data that show regulation by FapR (*psoA-psoE* and AfuB_086000). Deletion of the previously characterized *psoA* (26) also yielded loss of pseurotin in our study. Deletion of *psoC*, encoding a putative methyltransferase; *psoD*, encoding a putative P450 monooxygenase; and *psoE*, encoding a putative GST, each showed loss of pseurotin production, and, in the case of Δ*psoE*, accumulation of deepoxy-synerazol ($C_{22}H_{23}NO_6$ $[M + H]^+$, *m/z* 398.1598; found, *m/z* 398.1615). Deletion of *psoB*, encoding a putative hydrolase, resulted in significant reduction of pseurotin production, whereas deletion of the other gene encoding a putative hydrolase (AfuB_086000) did not alter pseurotin levels compared with the wild type (Table 1, Fig. S2, and Fig. S3). Because we found several genes between *fmaA* and *fmaB* not to participate in fumagillin production, we specifically analyzed these gene deletions for a possible role in pseurotin biosynthesis. Surprisingly, deletion of the genes *psoF* and *psoG*, encoding a putative enzyme with dual function as methyltransferase and monooxygenase and a hypothetical protein,

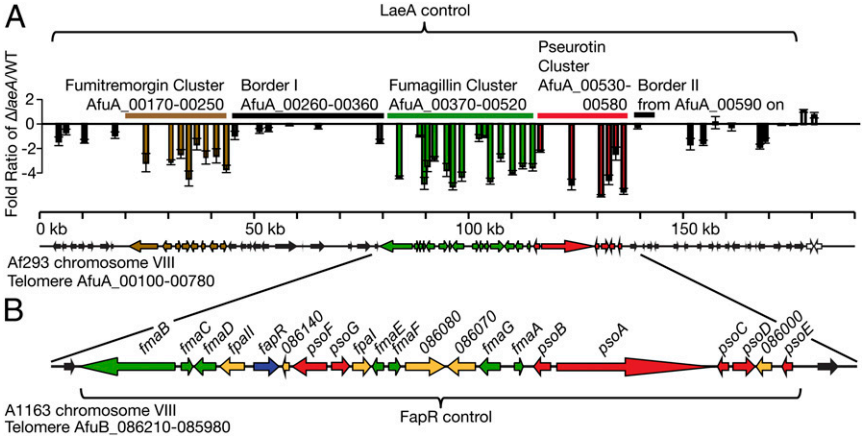

Fig. 1. Subtelomeric region of *A. fumigatus* chromosome VIII controlled by LaeA and FapR. (*A*) Schematic representation of *A. fumigatus* Af2931 genes AfuA_8g00100-AfuA_8g00780. Log_2 expression data from microarray experiments comparing *A. fumigatus* wild-type to Δ*laeA* gene expression, as performed by Perrin et al. (24). Fumitremorgin, fumagillin, and pseurotin cluster genes are color-coded based on literature data (see *Results*). (*B*) Schematic representation of *A. fumigatus* A1163 genes AfuB_086210-AfuB_085980. Black indicates genes not regulated by FapR; green indicates genes involved in fumagillin biosynthesis that are FapR-regulated; red indicates genes involved in pseurotin biosynthesis that are FapR-regulated; yellow indicates genes involved in neither fumagillin nor pseurotin biosynthesis but are FapR-regulated; blue indicates gene encoding FapR.

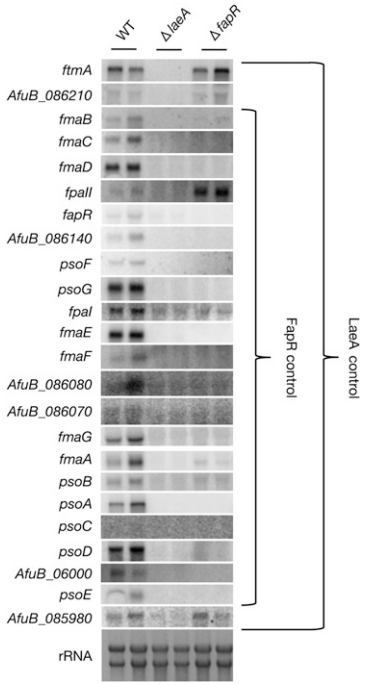

Fig. 2. Northern expression analysis of genes of the fumagillin and pseurotin supercluster and adjacent border genes. Northern expression analysis of indicated genes comparing the *A. fumigatus* wild type (CEA17), the Δ*laeA* mutant, and the Δ*fapR* mutant. Ribosomal RNA was visualized by ethidium bromide staining as loading control. The indicated strains were grown in liquid GMM at 25 °C and 250 rpm for 96 h under ambient light conditions.

respectively, originally predicted to be required for fumagillin synthesis, showed loss of pseurotin production. In the case of Δ*psoF*, we observed accumulation of demethyl-deepoxy-synerazol ($C_{21}H_{21}NO_6$ $[M + H]^+$, *m/z* 384.1442; found, *m/z* 384.1447) instead of pseurotin (Table 1, Fig. S2, and Fig. S3). Our data demonstrate that these genes, located in between fumagillin biosynthetic enzyme-encoding genes, participate in pseurotin production.

Rearrangement of the Supercluster in *N. fischeri*, *A. clavatus*, and *M. anisopliae*. BLAST analysis showed high similarity of some of the FapR-regulated genes to the closely related species *A. clavatus* NRRL 1, *N. fischeri* NRRL 181, and the entomopathogenic fungus *M. anisopliae* ARSEF 23 (Table S1). Thus, we analyzed the synteny of the fumagillin/pseurotin supercluster region in more detail comparing these four species. In *N. fischeri*, the left (*fmaB* to *fmaD*) and right (*psoE*) parts of the supercluster are syntenic to *A. fumigatus* and located in homologous genomic positions in both species. The region within the cluster edges shows evidence for gene-inversion and putative gene-loss events between *N. fischeri* and *A. fumigatus*. Most strikingly, the entire region present in *A. fumigatus* (including *fmaG*, *fmaA*, and *psoB* to *psoD*) is absent in *N. fischeri*. However, the region *psoB* to *psoD* is the only homologous region present with a high degree of synteny in *A. clavatus*, a species that appears to have lost the *fma* genes, as well as *fapR* (Fig. 3).

In the more distantly related species *M. anisopliae*, homologs of all genes, except for *fmaB*, *fmaC*, *fmaE*, are present in an intertwined supercluster region. However, the degree of synteny of the two superclusters is weak, showing a syntenic arrangement only for the region *psoB* to *psoD*, as also observed in *A. clavatus*. Interestingly, two genes inside the *A. fumigatus* supercluster, AfuB_086080 and AfuB_086000, which have no bidirectional best hit in the *M. anisopliae* genome, show a higher degree of similarity to parts of the respective *M. anisopliae* proteins, MAA_08355 and PsoA, than to homologs of these proteins in *A. fumigatus* (FpaII and PsoA, respectively) (Fig. 3 and Table S1).

Discussion

The opportunistic human pathogen *A. fumigatus* is known to produce at least 226 potentially bioactive secondary metabolites that can be grouped into 24 distinct biosynthetic families (30). The genome sequence is publicly available (29, 31, 32), and several metabolites have been linked to their key enzyme-encoding gene clusters (reviewed in ref. 33). All of these metabolites have been associated with physically distinct, single-locus gene clusters, predicted by secondary-metabolite cluster search algorithms that are based on the assumption that genes involved in the synthesis of a particular metabolite are contiguously aligned (22, 23). In the few cases where a metabolic pathway is split into two clusters [e.g., the trichothecene clusters (34)], these separated clusters are devoted to synthesis of one metabolite. In contrast, this work revealed the complex intertwining of fumagillin and pseurotin biosynthetic genes and, thus, identifies an example of a gene cluster encoding for two unrelated chemicals. Moreover, comparative genomics indicates this interwoven motif is conserved and belongs to a rapidly evolving region of diverse fungal genomes.

Hierarchical Levels of Cluster Regulation. LaeA, initially identified in *A. nidulans* as positive regulator of the $Zn(II)_2Cys_6$ pathway-specific sterigmatocystin regulator AflR (8), was shown to affect multiple known and cryptic secondary-metabolite gene clusters in *A. fumigatus* Af293 (24). The 170-kb subtelomeric region of chromosome VIII regulated by LaeA includes genes encoding the key enzymes for fumitremorgin, fumagillin, and pseurotin production.

To more thoroughly address the regulatory network of these three clusters, we deleted the only LaeA-regulated transcription factor-encoding gene in this region, *fapR*. Typically $Zn(II)_2Cys_6$ proteins are involved in the regulation of a single cluster (7); however, we found that FapR regulated both the fumagillin and pseurotin but not the fumitremorgin clusters, as assessed by transcriptional and biochemical analysis. The region controlled by FapR spans 21 consecutive genes, including previously characterized *fmaA*, *fmaB*, *fmaC*, and *psoA*. In contrast, the region regulated by LaeA extends past the FapR-regulated region on both sides, resulting in additional down-regulation of *ftm* cluster genes in the Δ*laeA* mutant compared with the wild type and the Δ*fapR* mutant (Fig. 1, Fig. 2, Fig. S1, and Table 1).

Biosynthetic Genes of Two Clusters Are Interwoven. Because deletion of *fapR* resulted in loss of both fumagillin and pseurotin production, we deleted all genes regulated by FapR in the supercluster, as well as border genes, to delineate the two independent biosynthetic pathways. As expected, gene deletions of the border genes did not alter fumagillin and pseurotin production. The analysis of the FapR-regulated gene mutants yielded the unexpected finding that the fumagillin and pseurotin cluster genes were intertwined in one supercluster. Specifically, we found two genes, *psoF* and *psoG*, that are located between the previously characterized fumagillin key enzyme-encoding genes *fmaA* and *fmaB* and predicted to be part of the fumagillin cluster, to actually be involved in pseurotin biosynthesis (Fig. 4, Fig. S2, and Table 1).

Our results regarding putative intermediates in the deletion mutants obtained in this study essentially support the pathways suggested for fumagillin and pseurotin by Lin et al. (27) and Maiya et al. (26), respectively (Fig. 4 and Fig. S3). The data obtained in this study suggest that the putative *O*-methyltransferase FmaD is responsible for methylation of the C6-hydroxyl group of β-*trans*-bergamotene during fumagillin biosynthesis. *O*-methyltransferases in a variety of secondary-metabolite gene clusters in different fungal species have been demonstrated to catalyze similar reactions (35–37). Identification of a fumagillin-aldehyde derivative in the Δ*fmaF* mutant suggests that the putative phytanoyl-CoA-oxidase is involved in the final oxidation step of the tetraene chain. Regarding pseurotin biosynthesis, we were able to identify deepoxy-synerazol in mutants lacking the putative GST, PsoE, suggesting its involvement in oxidation of the diene chain.

Table 1. Chemical analysis of strains used in this study

Strain	Fumitremorgin	Fumagillin	Pseurotin
CEA17 KU80Δ (WT)	Yes	Yes	Yes
Δ*laeA*	No	No	No
Δ*6270–10*	Yes	Yes	Yes
Δ*fmaB*	Yes	Fumagillol	Yes
Δ*fmaC*	Yes	Fumagillol	Yes
Δ*fmaD*	Yes	Demethyl-fumagillin	Yes
Δ*fpaII*	Yes	Yes	Yes
Δ*fapR*	Yes	No	No
Δ*6140*	Yes	Yes	Yes
Δ*psoF*	Yes	Yes	Demethyl-deepoxy-synerazol
Δ*psoG*	Yes	Yes	No
Δ*fpaI*	Yes	Yes	Yes
Δ*fmaE*	Yes	No	Yes
Δ*fmaF*	Yes	Dimethyl-fumagillin-aldehyde	Yes
Δ*6080*	Yes	Yes	Yes
Δ*6070*	Yes	Yes	Yes
Δ*fmaG*	Yes	No	Yes
Δ*fmaA*	Yes	No	Yes
Δ*psoB*	Yes	Yes	No
Δ*psoA*	Yes	Yes	No
Δ*psoC*	Yes	Yes	No
Δ*psoD*	Yes	Yes	No
Δ*6000*	Yes	Yes	Yes
Δ*psoE*	Yes	Yes	Deepoxy-synerazol
Δ*5980*	Yes	Yes	Yes

Furthermore, the identification of demethyl-deepoxy-synerazol in the Δ*psoE* mutant argues for the putative dual-functional monooxygenase/methyltransferase PsoE to catalyze an *O*-methylation at C8 previous to oxidation of the diene chain by PsoF. Notably, a truncated version of PsoE was shown to catalyze a Baeyer–Villiger oxidation in vitro (38). However, our results do not support a role in oxidation during pseurotin biosynthesis in vivo. There is precedence for a putative dual-functional monooxygenase/methyltransferase in the biosynthesis of cercosporin in *Cercospora nicotianae* (39), but the exact reaction mechanism is unresolved to date. The remaining unresolved biosynthetic steps in both pathways are likely to be catalyzed by the enzymes whose gene deletions led to loss of end products, but no intermediate could be identified (possibly because of instability of those compounds). The putative P450 monooxygenases FmaG and PsoD could participate at multiple oxidation steps during fumagillin and pseurotin biosynthesis, respectively. In fungal species, there is precedence for P450 to promiscuously participate at multiple oxidation steps at varying carbon atoms (40). A homolog of the putative hydrolase PsoB was recently shown to be involved in 2-pyrrolidine ring closure during fusarin biosynthesis (41), making a similar role for PsoB during pseurotin biosynthesis feasible (Fig. 4 and Fig. S2). The roles of the hypothetical proteins FmaE, PsoG, and the putative methyltransferase PsoC during fumagillin and pseurotin biosynthesis, respectively, remain to be elucidated.

Conserved Interwoven Supercluster Region. Bioinformatic analysis of the fumagillin/pseurotin supercluster region revealed that it is maintained despite several gene-rearrangement and gene-loss events in the closely related species *N. fischeri* NRRL 181 and *A. clavatus* NRRL 1, as well as the distantly related species *M. anisopliae* ARSEF 23 (Fig. 3). Although there is no report of pseurotin or fumagillin production in *N. fischeri*, several *Neosartorya* spp. have been reported to produce these or similar metabolites (42, 43), presumably because of strain-specific differences in the specific fumagillin/pseurotin supercluster region. Because a different *Metarhizium* sp. was reported to produce the structurally related fumagillol compound, hydroxy-ovalicin (44), it is most likely that the genes required for its biosynthesis are encoded in the fumagillin/pseurotin-like supercluster region identified in this study. We note that fumagillin and pseurotin production, or variations thereof, have also been reported in *Penicillium* spp. (45). Coupled with our findings here, we suggest that a certain degree of evolutionary pressure has maintained this interwoven supercluster motif in diverse fungi and speculate

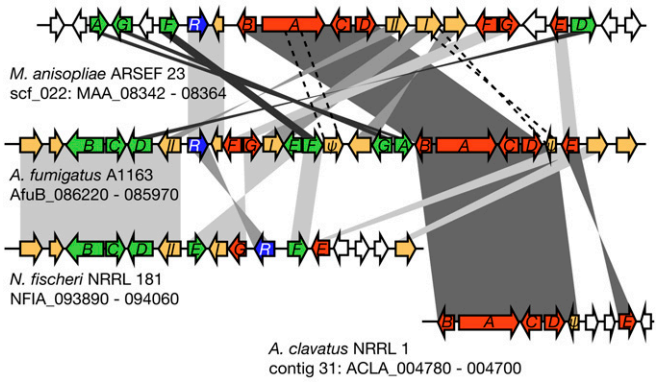

Fig. 3. Comparison of the fumagillin/pseurotin supercluster region in *A. fumigatus*, *A. clavatus*, *N. fischeri*, and *M. anisopliae*. The genes involved in fumagillin biosynthesis are represented as green arrows. ("A" through "G" in green arrows indicate *fmaA* to *-G*.) The genes involved in pseurotin biosynthesis are represented as red arrows. ("A" through "G" in red arrows indicate *psoA* to *-G*.) Dark blue arrow depicts the genes encoding FapR. The yellow arrows represent genes with homologs in *N. fischeri* and/or *M. anisopliae* in the immediate supercluster region that do not participate in fumagillin or pseurotin biosynthesis; ψ marks pseudogenes that show no bidirectional best hit in the immediate homologous supercluster region of either *N. fischeri* or *M. anisopliae*. White arrows represent genes in *F. fischeri*, *M. anisopliae*, and *A. clavatus* with no bidirectional best hit to the immediate homologous supercluster region of each other or *A. fumigatus*. Connecting gray rectangles indicate homologies among the species. Blue arrows with "R" indicate *fapR* (fumagillin and pseurotin regulator); orange arrows with "I" indicate *fpaI*; orange arrows with "II" indicate *fpaII*.

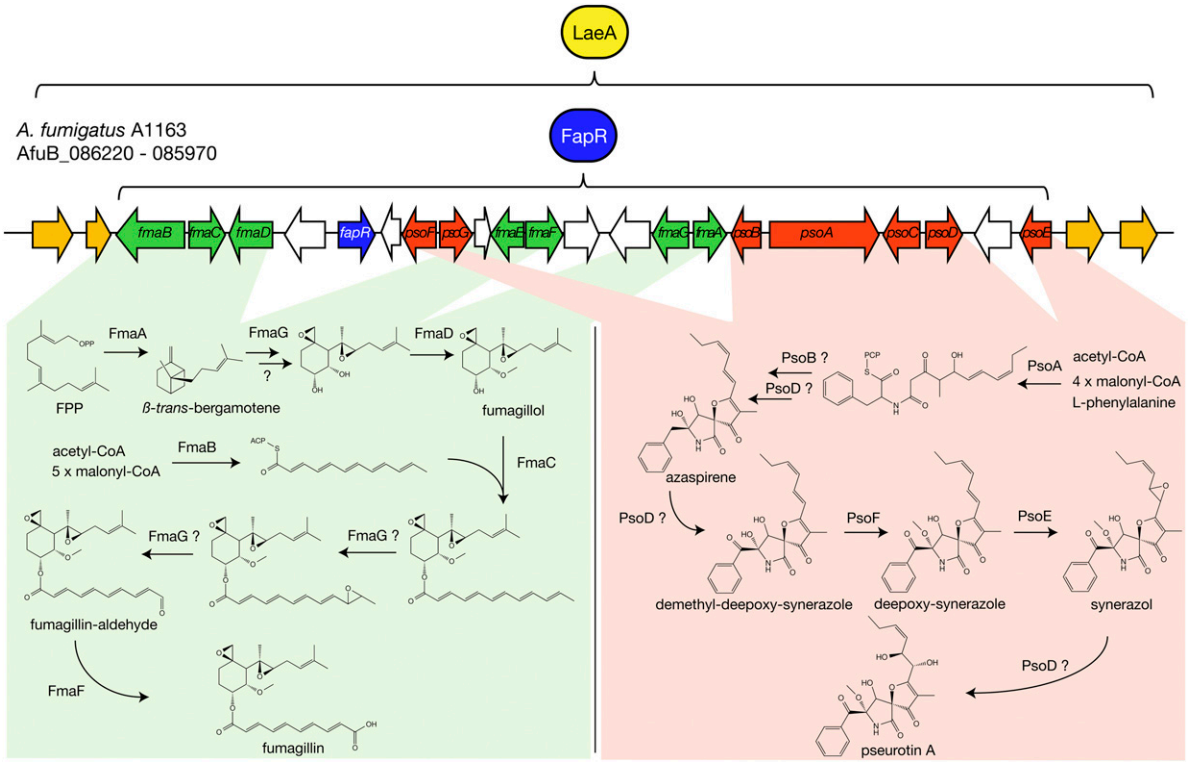

Fig. 4. Proposed biosynthetic pathways of fumagillin and pseurotin and regulation mechanism of the participating genes by LaeA and FapR. Green arrows represent genes involved in fumagillin biosynthesis. Red arrows represent genes involved in pseurotin biosynthesis. White arrows represent genes that are regulated by FapR and LaeA but not involved in either fumagillin or pseurotin production. Orange arrows represent supercluster border genes regulated by LaeA but not FapR and not involved in fumagillin or pseurotin biosynthesis. The dark blue arrow represents the gene encoding FapR. ACP, acyl-carrier protein domain; FmaA, terpene cyclase involved in β-*trans*-bergamotene production; FmaG, P450 monooxygenase involved in oxidative rearrangement of β-*trans*-bergamotene; FmaD, *O*-methyltransferase involved in methylation of C4 hydroxyl group; FmaB and -C, PKS and accessory enzyme involved in pentaene chain production and connection to fumagillol, respectively; FmaF, phytanoyl-CoA oxidase involved in oxidation of fumagillin-aldehyde; FPP, farnesyl-pyrophosphate; PCP, peptidyl-carrier protein domain; PsoA, PKS/NRPS hybrid catalyzing formation of the pseurotin scaffold; PsoF, *O*-methyltransferase involved in methylation of the C8 hydroxyl group of azaspirene; PsoE, GST participating in oxidation of the diene chain. Question marks indicate speculative enzyme assignments.

that synergistic effects of the two compounds convey ecological fitness traits to the producing fungi. Additional sequence information of homologous clusters in different species will help determine whether this highly mobile supercluster is assembling by gene relocations of two formerly independent clusters or diverging into two independent clusters from one common ancestral supercluster.

This work demonstrates an additional layer of complexity in fungal secondary-metabolite gene clusters. Until now, biosynthetic genes contiguously aligned in a cluster have been associated with the production of a distinct natural product (and its intermediates). Previous to our work, the pseurotin and fumagillin clusters had been incorrectly annotated based on physical gene locations within the genomic context. In contrast to this view of stand-alone secondary-metabolite clusters, our collective data have revealed the existence of superclusters with interwoven biosynthetic genes involved in formation of more than one chemical product. Comparison with other fungal species demonstrates that despite several rearrangement events, similar intertwined cluster motifs are maintained in related clusters.

Materials and Methods

Fungal Strains, Culture Conditions, and HPLC-MS Analyses. *A. fumigatus* strains used in this study are listed in Table S2. Strains were maintained as glycerol stocks and activated on solid glucose minimal medium (GMM) at 37 °C with appropriate supplements (46). For *pyrG* auxotrophs, the growth medium was supplemented with 5 mM uridine and uracil, respectively. Conidia were harvested in 0.01% Tween 80 and enumerated using a hemacytometer. For fumagillin, pseurotin, and fumitremorgin product and gene expression analysis, the strains were inoculated into liquid GMM at 5×10^6 conidia per milliliter and grown at 25 °C and 250 rpm for 96 h in ambient light conditions. The liquid medium was then lyophilized and redissolved in 25 mL double-distilled H_2O. The water crude was partitioned with 25 mL of ethyl acetate (EA). The EA layer was evaporated in vacuo and redissolved in 1 mL of 20% (vol/vol) DMSO/MeOH, and a 10-μL portion was examined by HPLC-diode array detector-MS analysis (Figs. S3 and S4). HPLC-MS was carried out using a ThermoFinnigan LCQ Advantage ion-trap mass spectrometer with a reverse-phase C18 column (Alltech Prevail C18; 2.1 mm × 100 mm with a 3-μm particle size) at a flow rate of 125 μL/min. The solvent-gradient system for HPLC and the conditions for MS analysis were carried out as described previously (47).

Genetic Manipulation. The *A. fumigatus* mutant strains were constructed using a double-joint fusion PCR approach (48), and all primers used in the study are listed in Table S3. Briefly, ~1-kb fragments flanking the targeted deletion region were amplified by PCR from *A. fumigatus* strain CEA17 genomic DNA, and the *Aspergillus parasiticus pyrG* marker gene was amplified from the plasmid pJW24 (49). The three fragments were subjected to fusion PCR to generate deletion cassettes (for details, see *SI Text*). Genes were deleted in *A. fumigatus* CEA17KU80 *pyrG*$^-$ strain (50) (Table S2). Single integration of the transformation construct was confirmed by Southern analysis using P^{32}-labeled probes created by amplification of the respective deletion construct using primer pair [gene]-5F/[gene]-3R (Table S3 and Fig. S5).

RNA Extraction and Northern Analysis. Mycelia were harvested by filtration through Miracloth (Calbiochem). Total RNA was extracted with TRIzol reagent (Invitrogen) from freeze-dried mycelia, following the manufacturer's protocol. Probes for Northern analysis were constructed at regions internal

to the gene of interest using primers listed in Table S3 ([gene]-F/[gene]-R) and labeled with dCTP αP^{32}.

Sequence Analysis. Sequences for *A. fumigatus* A1163 and Af293, as well as *N. fischeri* NRRL 181, were retrieved from the *Aspergillus* genome database AspGD (www.aspergillusgenome.org) (51). *M. anisopliae* ARSEF 23 sequences were retrieved from the published genome sequence (52). Sequence-homology searches were performed using the National Center for Biotechnology Information database server and BLAST algorithm (53). Proteins from two species were regarded as bidirectional best hits when they produced reciprocal highest identities (*e* value, $\leq 1 \times e^{-10}$) in either BLASTP or BLASTX analysis.

Isolation and Characterization of Secondary Metabolites. For structure elucidation, fumagillin was isolated from 4 L of GMM liquid medium cultivated with strain TPHW7.1 under the aforementioned culture condition as described above. Pseurotin A was isolated from 4 L of GMM liquid culture of strain TPHW4.4. The UV-active secondary metabolites were isolated via flash chromatography and reverse-phase HPLC. A portion of 10 μL of each purified compound solution from the reverse-phase HPLC was then examined by an Agilent Technologies 1200 series high-resolution mass spectrometer. IR spectra were recorded on a GlobalWorks Cary 14 Spectrophotometer. NMR spectra were collected on a Varian Mercury Plus 400 spectrometer (for details, see Table S4 and *SI Text*).

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1. Hoffmeister D, Keller NP (2007) Natural products of filamentous fungi: Enzymes, genes, and their regulation. *Nat Prod Rep* 24(2):393–416.
2. Brakhage AA (2013) Regulation of fungal secondary metabolism. *Nat Rev Microbiol* 11(1):21–32.
3. Rohlfs M, Churchill AC (2011) Fungal secondary metabolites as modulators of interactions with insects and other arthropods. *Fungal Genet Biol* 48(1):23–34.
4. Eisenman HC, Casadevall A (2012) Synthesis and assembly of fungal melanin. *Appl Microbiol Biotechnol* 93(3):931–940.
5. Smith DJ, et al. (1990) Beta-lactam antibiotic biosynthetic genes have been conserved in clusters in prokaryotes and eukaryotes. *EMBO J* 9(3):741–747.
6. Schjerling P, Holmberg S (1996) Comparative amino acid sequence analysis of the C6 zinc cluster family of transcriptional regulators. *Nucleic Acids Res* 24(23):4599–4607.
7. Chang PK, Ehrlich KC (2013) Genome-wide analysis of the $Zn(II)_2Cys_6$ zinc cluster-encoding gene family in Aspergillus flavus. *Appl Microbiol Biotechnol* 97(10):4289–4300.
8. Bok JW, Keller NP (2004) LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryot Cell* 3(2):527–535.
9. Bok JW, et al. (2005) LaeA, a regulator of morphogenetic fungal virulence factors. *Eukaryot Cell* 4(9):1574–1582.
10. Bayram Ö, et al. (2008) VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science* 320(5882):1504–1506.
11. Amaike S, Keller NP (2009) Distinct roles for VeA and LaeA in development and pathogenesis of *Aspergillus flavus*. *Eukaryot Cell* 8(7):1051–1060.
12. Kosalková K, et al. (2009) The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in *Penicillium chrysogenum*. *Biochimie* 91(2):214–225.
13. Wiemann P, et al. (2010) FfVel1 and FfLae1, components of a *velvet*-like complex in *Fusarium fujikuroi*, affect differentiation, secondary metabolism and virulence. *Mol Microbiol* 77(4):972–994.
14. Hoff B, et al. (2010) Two components of a velvet-like complex control hyphal morphogenesis, conidiophore development, and penicillin biosynthesis in *Penicillium chrysogenum*. *Eukaryot Cell* 9(8):1236–1250.
15. Wu D, Oide S, Zhang N, Choi MY, Turgeon BG (2012) ChLae1 and ChVel1 regulate T-toxin production, virulence, oxidative stress response, and development of the maize pathogen *Cochliobolus heterostrophus*. *PLoS Pathog* 8(2):e1002542.
16. Karimi-Aghcheh R, et al. (2013) Functional analyses of *Trichoderma reesei* LAE1 reveal conserved and contrasting roles of this regulator. *G3 (Bethesda)* 3(2):369–378.
17. Bok JW, Noordermeer D, Kale SP, Keller NP (2006) Secondary metabolic gene cluster silencing in *Aspergillus nidulans*. *Mol Microbiol* 61(6):1636–1645.
18. Lim FY, et al. (2012) Genome-based cluster deletion reveals an endocrocin biosynthetic pathway in *Aspergillus fumigatus*. *Appl Environ Microbiol* 78(12):4117–4125.
19. Butchko RA, Brown DW, Busman M, Tudzynski B, Wiemann P (2012) Lae1 regulates expression of multiple secondary metabolite gene clusters in *Fusarium verticillioides*. *Fungal Genet Biol* 49(8):602–612.
20. Bouhired S, Weber M, Kempf-Sontag A, Keller NP, Hoffmeister D (2007) Accurate prediction of the *Aspergillus nidulans* terrequinone gene cluster boundaries using the transcriptional regulator LaeA. *Fungal Genet Biol* 44(11):1134–1145.
21. Yin WB, et al. (2013) A nonribosomal peptide synthetase-derived iron(III) complex from the pathogenic fungus Aspergillus fumigatus. *J Am Chem Soc* 135(6):2064–2067.
22. Khaldi N, et al. (2010) SMURF: Genomic mapping of fungal secondary metabolite clusters. *Fungal Genet Biol* 47(9):736–741.
23. Medema MH, et al. (2011) antiSMASH: Rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic Acids Res* 39(Web Server issue):W339–W346.
24. Perrin RM, et al. (2007) Transcriptional regulation of chemical diversity in *Aspergillus fumigatus* by LaeA. *PLoS Pathog* 3(4):e50.
25. Maiya S, Grundmann A, Li SM, Turner G (2006) The fumitremorgin gene cluster of *Aspergillus fumigatus*: Identification of a gene encoding brevianamide F synthetase. *ChemBioChem* 7(7):1062–1069.
26. Maiya S, Grundmann A, Li X, Li SM, Turner G (2007) Identification of a hybrid PKS/NRPS required for pseurotin A biosynthesis in the human pathogen *Aspergillus fumigatus*. *ChemBioChem* 8(14):1736–1743.
27. Lin HC, et al. (2013) The fumagillin biosynthetic gene cluster in *Aspergillus fumigatus* encodes a cryptic terpene cyclase involved in the formation of *β-trans*-bergamotene. *J Am Chem Soc* 135(12):4616–4619.
28. Li SM (2011) Genome mining and biosynthesis of fumitremorgin-type alkaloids in ascomycetes. *J Antibiot (Tokyo)* 64(1):45–49.
29. Fedorova ND, et al. (2008) Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*. *PLoS Genet* 4(4):e1000046.
30. Frisvad JC, Rank C, Nielsen KF, Larsen TO (2009) Metabolomics of *Aspergillus fumigatus*. *Med Mycol* 47(Suppl 1):S53–S71.
31. Galagan JE, et al. (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* 438(7071):1105–1115.
32. Nierman WC, et al. (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* 438(7071):1151–1156.
33. Inglis DO, et al. (2013) Comprehensive annotation of secondary metabolite biosynthetic genes and gene clusters of *Aspergillus nidulans, A. fumigatus, A. niger* and *A. oryzae*. *BMC Microbiol* 13:91.
34. Proctor RH, McCormick SP, Alexander NJ, Desjardins AE (2009) Evidence that a secondary metabolic biosynthetic gene cluster has grown by gene relocation during evolution of the filamentous fungus *Fusarium*. *Mol Microbiol* 74(5):1128–1142.
35. Keller NP, Dischinger HC, Jr., Bhatnagar D, Cleveland TE, Ullah AH (1993) Purification of a 40-kilodalton methyltransferase active in the aflatoxin biosynthetic pathway. *Appl Environ Microbiol* 59(2):479–484.
36. Wiemann P, et al. (2009) Biosynthesis of the red pigment bikaverin in *Fusarium fujikuroi*: Genes, their function and regulation. *Mol Microbiol* 72(4):931–946.
37. Ali H, et al. (2013) A branched biosynthetic pathway is involved in production of roquefortine and related compounds in *Penicillium chrysogenum*. *PLoS ONE* 8(6):e65328.
38. Mascotti ML, Juri Ayub M, Dudek H, Sanz MK, Fraaije MW (2013) Cloning, overexpression and biocatalytic exploration of a novel Baeyer-Villiger monooxygenase from *Aspergillus fumigatus* Af293. *AMB Express* 3(1):33.
39. Dekkers KL, et al. (2007) The *Cercospora nicotianae* gene encoding dual *O*-methyltransferase and FAD-dependent monooxygenase domains mediates cercosporin toxin biosynthesis. *Fungal Genet Biol* 44(5):444–454.
40. Bömke C, Tudzynski B (2009) Diversity, regulation, and evolution of the gibberellin biosynthetic pathway in fungi compared to plants and bacteria. *Phytochemistry* 70(15-16):1876–1893.
41. Niehaus EM, et al. (2013) Multifactorial manipulation of the *Fusarium fujikuroi* fusarin gene cluster yields insight into the complex regulation and fusarin biosynthetic pathway. *Chem Biol* 20(8):1055–1066.
42. Asami Y, et al. (2002) Azaspirene: A novel angiogenesis inhibitor containing a 1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione skeleton produced by the fungus *Neosartorya* sp. *Org Lett* 4(17):2845–2848.
43. Asami Y, et al. (2004) RK-805, an endothelial-cell-growth inhibitor produced by *Neosartorya* sp., and a docking model with methionine aminopeptidase-2. *Tetrahedron* 60(33):7085–7091.
44. Kuboki H, et al. (1999) Mer-f3, 12-hydroxy-ovalicin, produced by Metarrhizium sp. f3. *J Antibiot (Tokyo)* 52(6):590–593.
45. Christensen M, Frisvad JC, Tuthill D (1999) Taxonomy of the *Penicillium miczynskii* group based on morphology and secondary metabolites. *Mycol Res* 103(5):527–541.
46. Shimizu K, Keller NP (2001) Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. *Genetics* 157(2):591–600.
47. Guo CJ, et al. (2013) Biosynthetic pathway for the epipolythiodioxopiperazine acetylaranotin in *Aspergillus terreus* revealed by genome-based deletion analysis. *J Am Chem Soc* 135(19):7205–7213.
48. Szewczyk E, et al. (2006) Fusion PCR and gene targeting in *Aspergillus nidulans*. *Nat Protoc* 1(6):3111–3120.
49. Calvo AM, Bok J, Brooks W, Keller NP (2004) *veA* is required for toxin and sclerotial production in *Aspergillus parasiticus*. *Appl Environ Microbiol* 70(8):4733–4739.
50. da Silva Ferreira ME, et al. (2006) The *akuB*(^{KU80}) mutant deficient for nonhomologous end joining is a powerful tool for analyzing pathogenicity in *Aspergillus fumigatus*. *Eukaryot Cell* 5(1):207–211.
51. Arnaud MB, et al. (2012) The *Aspergillus* Genome Database (AspGD): Recent developments in comprehensive multispecies curation, comparative genomics and community resources. *Nucleic Acids Res* 40(Database issue):D653–D659.
52. Gao Q, et al. (2011) Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. *PLoS Genet* 7(1):e1001264.
53. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215(3):403–410.