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Genomic Mining for *Aspergillus* Natural Products

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Summary

The genus *Aspergillus* is renowned for its ability to produce a myriad of bioactive secondary metabolites. Although the propensity of biosynthetic genes to form contiguous clusters greatly facilitates assignment of putative secondary metabolite genes in the completed *Aspergillus* genomes, such analysis cannot predict gene expression and, ultimately, product formation. To circumvent this deficiency, we have examined *Aspergillus nidulans* microarrays for expressed secondary metabolite gene clusters by using the transcriptional regulator LaeA. Deletion or overexpression of *laeA* clearly identified numerous secondary metabolite clusters. A gene deletion in one of the clusters eliminated the production of the antitumor compound terrequinone A, a metabolite not described, from *A. nidulans*. In this paper, we highlight that LaeA-based genome mining helps decipher the secondary metabolome of Aspergilli and provides an unparalleled view to assess secondary metabolism gene regulation.

Introduction

Filamentous fungi display many unique characteristics that render them of great interest to the research community. Among these characteristics is the biosynthesis of natural products that display a broad range of useful activities for pharmaceutical and agricultural purposes, e.g., antibiotic, immunosuppressant, lipid-lowering, or antifungal properties [1–3]. Less desired are the potent phyto- and mycotoxic activities exhibited by fungal pathogens [4, 5]. These bioactivities of natural products have spurred efforts toward identifying genes involved in their biosynthesis. Accumulating data from studies of known secondary metabolite biosynthetic genes dispelled an original premise that fungal metabolic genes would be scattered throughout the genome; rather, the hallmark of secondary metabolite genes—in contrast to genes involved in primary metabolism—is that they are clustered in fungal genomes [6]. Examples of secondary metabolite gene clusters include those synthesizing pharmaceuticals of clinical use, such as the important β-lactam antibiotics penicillin (PN) and cephalosporin [7], the antihypercholesterolaemic agent lovastatin [8], the ergopeptines with their important pharmacophore D-lysergic acid amide [9], as well as carcinogenic toxins (aflatoxin and sterigmatocystin, ST) [4].

Discovery of the first fungal gene clusters was largely a result of mutant hunts, followed by complementation with gene transformation. More sophisticated identification techniques arose when it was realized that many of the structural genes involved in secondary metabolism are highly conserved and could be cloned by hybridization probing or amplified from the fungal genome by use of degenerate primers; the latter technique was especially fruitful in procuring polyketide synthases [10, 11]. This conservation of DNA and protein sequences, coupled with the cluster motif of metabolic pathways, greatly facilitated the assignment of putative secondary metabolite genes in the completed *Aspergillus* genomes (Broad Institute, Sanger Centre, and TIGR). Sequence alignments suggest that *A. nidulans* has the potential to generate up to 27 polyketides, 14 nonribosomal peptides, 1 terpene, and 2 indole alkaloids; similar predictions can be made from the *A. fumigatus* and *A. oryzae* genomes. Interestingly, there appears to be almost no orthologs among these genes across the three species, thus representing a loss of synteny to a degree not seen in other regions of the genomes. This high number of putative metabolites is greater than the known metabolites ascribed to these species, and it may be a reflection of incomplete natural product analysis in these species or failure of many clusters to be expressed, at least under the culture conditions commonly used in laboratories. For example, the aflatoxin gene cluster is not transcribed in *A. oryzae* [6, 12].

An intimation of a method to identify transcriptionally active clusters arose from the discovery of LaeA, a nuclear protein regulating secondary metabolite production in *Aspergillus* spp. [13]. Loss of LaeA silenced ST and PN production in *A. nidulans* and gliotoxin production in *A. fumigatus*, whereas overexpression of the gene increased PN and lovastatin production in *A. nidulans* and *A. terreus*, thus leading to the hypothesis that LaeA was involved in global regulation of secondary metabolite gene clusters in this genus. This differs from the *Streptomyces* transcriptional regulator AfsR, which is either species or pathway specific [14, 15]. Given the transcriptional nature of regulation by LaeA, we considered this protein to be a promising gateway toward designing a novel procedure to identify fungal natural products. Our prediction, realized here, was that any

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transcribed secondary metabolite cluster in *Aspergillus* would be revealed by profiling *laeA* deletion (*ΔlaeA*) and overexpression (*OE::laeA*) mutants. Profiling allows for the identification of transcriptionally active clusters, targeted manipulation, and, ultimately, chemical characterization of novel natural products. As proof of principle, we present here the LaeA-based identification of the terrequinone biosynthetic gene cluster in the *Aspergillus nidulans* genome, an antitumor compound previously unknown from this species.

Results and Discussion

Two of the best-characterized fungal secondary metabolite gene clusters are the *A. nidulans* ST and PN clusters. Prior gene expression data of the *A. nidulans ΔlaeA* strain compared to wild-type showed that selected genes in the ST and PN gene clusters were downregulated in the mutant [13]. To examine the nature and extent of ST and PN gene cluster regulation by LaeA, we analyzed a full genome array for ST and PN gene expression. Figure 1 illustrates the log ratios comparing expression of genes (*ΔlaeA* versus wild-type) of the ST and PN gene clusters. The pattern produced is what we term the "secondary metabolite cluster signature," in which individual genes or even virtually every gene in the particular cluster is downregulated in the *ΔlaeA* strain, in contrast to the undisturbed expression of adjacent genes. To validate these results, we assessed a transcriptional profile of the entire 60 kb ST gene cluster by Northern analysis (data not shown). The profile was remarkably similar to that of the array and confirmed that LaeA regulation impacts the cluster region, but not neighboring genes.

Considering the clear presentation of the secondary metabolism motif for the PN and ST clusters, we then examined the array data for areas of near-contiguous gene suppression in the *ΔlaeA* strain or near-contiguous gene induction in the *OE::laeA* strain. Open reading frames found in regions displaying this motif were then examined for the potential to encode secondary metabolite biosynthetic enzymes. Using these criteria, we identified several putative secondary metabolite cluster signatures; one of these signatures is illustrated from both the *ΔlaeA* and *OE::laeA* comparisons to wild-type (Figure 2). At times, data from *OE::laeA* and *ΔlaeA* comparisons to wild-type were even supportive for both positive and negative regulation, respectively, of the same cluster (one example is shown in Figure S1; see the Supplemental Data available with this article online).

Among the biosynthetic loci identified by the *laeA*-based genome mining approach for natural product biosynthetic capabilities, one particular locus whose secondary metabolite cluster signature is shown in Figure 2A attracted our attention. It comprises five open reading frames, transcriptionally regulated by LaeA (Figure 3A), two of which are similar to genes encoding transferases acting on tryptophan-derived structures (*tdiB*, dimethylallyl-L-tryptophan synthase, and *tdiD*, L-kynurenine aminotransferase, respectively), thus being suggestive of indole alkaloid biosynthetic abilities. Other reading frames encode for one hypothetical fungal protein (*tdiE*), one dehydrogenase/oxidoreductase (*tdiC*), and a monomodular nonribosomal peptide synthetase (NRPS, *tdiA*). Interestingly, the deduced TdiA enzyme significantly deviates from the canonical NRPS architecture [16, 17], as it merely comprises an adenylation domain and a peptidyl carrier domain, but lacks a condensation domain to form a peptide bond. However, it includes a thioesterase domain that releases the completed peptide from the enzyme, which is typically a bacterial NRPS feature [18]. The highest similarity across the entire deduced amino acid sequence was found to putative NRPSs of bacterial origin (*Ralstonia solanacearum* and *Burkholderia pseudomallei*, ENTREZ accession numbers NP_522978 and YP_110151, respectively). These findings suggest either an enzyme acting in *trans* on a second condensing NRPS encoded outside the cluster, a solely adenylating (i.e., activating) enzyme, or a nonfunctional pseudogene, perhaps as a remnant of an ancient horizontal gene transfer.

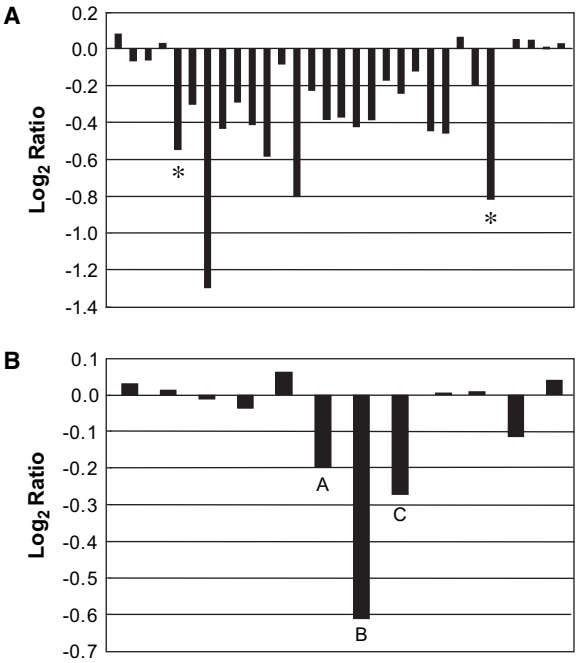


Figure 1. Genome Mining Identifies Previously Identified Gene Clusters of the LaeA Regulon

(A) Sterigmatocystin (ST) gene cluster. Shown are expression ratios (*ΔlaeA* to wild-type) for genes on Chromosome IV in the region including the ST cluster (AN7800.2–AN7830.2). Asterisks indicate the first (AN7804.2, *stcW*) and last (AN7825.2, *stcA*) genes of the cluster, relative to the genome sequence annotation.

(B) Penicillin (PN) gene cluster. Shown are expression ratios (*ΔlaeA* to wild-type) for genes on Chromosome II in the region including the PN cluster (AN2616.2–AN2627.2). PN genes *acvA*, *ipnA*, and *aatA* are indicated (A–C, respectively).

To determine if the cluster produced a bona fide secondary metabolite, we inactivated the second gene in the cluster, *tdiB. tdiB* shows similarity to *Claviceps fusiformis dmaW*, a gene encoding dimethylallyl-L-tryptophan synthase [19], and other fungal L-tryptophan dimethylallyltransferases, catalyzing the first committed step in the ergot alkaloid pathway. Similar to other secondary metabolite genes [13], *tdiB* is not only repressed in the *ΔlaeA* mutant, but it is also upregulated in a *laeA* overexpression background (Figure 3B). Disruption of *tdiB* resulted in a mutant (TJW65.7) unable to produce a

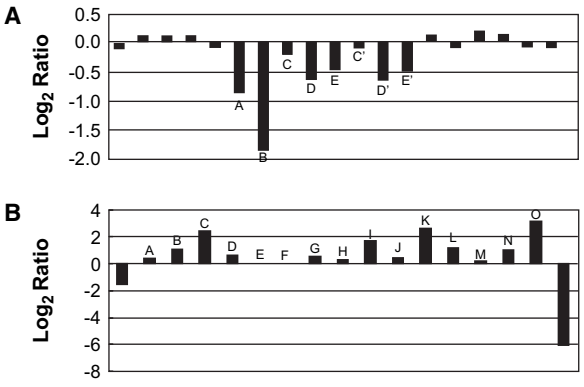


Figure 2. LaeA-Controlled Gene Clusters Identified by Genome Mining

(A) Putative indole alkaloid biosynthetic pathway. Shown are expression ratios (*ΔlaeA* to wild-type) for genes AN8513.2–AN8526.2. Genes belonging to the cluster (confirmed by Northern analysis, see text) putatively encode: (A) an NRPS possessing a peptidyl carrier protein, an adenylating domain, and a thioesterase domain, (B) tryptophan dimethylallyltransferase, (C) an dehydrogenase/oxidoreductase, (D) a homolog of kynurenine aminotransferase, and (E) a hypothetical protein. Genes C–E are duplicated in the annotated genome sequence (indicated as C′–E′), but this duplication does not exist in the fungal genome, as assessed by PCR.

(B) Putative hybrid secondary metabolite pathway. Shown are expression ratios (*OE::laeA* to wild-type) for genes AN1588.2–AN1602.2. Genes putatively belonging to the cluster include those encoding: (A–C) hypothetical proteins, (D) a putative ATPase family protein, (E) a polyprenyl synthase, (F) a hydroxylmethylglutaryl-coA reductase homolog (e^{-120}), (G) an ent-copalyl diphosphate/ent-kaurene synthase homolog (e^{-168}), (H) a translation elongation factor, (I) an oxidoreductase, (J) a hypothetical protein, (K) a P450 monooxygenase, (L) a Zn_2-Cys_6 transcription factor, (M) a hypothetical protein, (N) a cytochrome P450, and (O) a hypothetical protein.

compound that appears yellow-orange on TLC under UV light (Figure 4A). HPLC-UV/Vis and LC/MS analyses with extracts of the TJW65.7 mutant and wild-type identified ST as a known compound from *A. nidulans* in both samples (Figure 4B). However, the *ΔtdiB* sample was lacking a second major substance (Figure 4C). This compound was purified from the wild-type and was assigned a molecular mass of m/z = 490 (m/z = 489 [M − H^+] and 491 [M + H^+]). Full one- and two-dimensional NMR analyses (see Table S1 for details) identified the compound as terrequinone A (Figure 4D), a fungal bisindolylquinone with inhibitory properties on tumor cell lines [20], which was not known to be produced by *Aspergillus nidulans*.

Although feeding experiments have led to proposed biosynthetic routes for this class of compounds [21], gene clusters have not been identified for these metabolites. Matching the chemical structure of terrequinone A to the *tdi* cluster explains the absence of a condensation domain within the TdiA enzyme, as no amide bond has to be closed, and implicates a speculative, yet plausible, order for the key biosynthetic events: (1) deamination of L-tryptophan to indolepyruvic acid by the transaminase TdiD; (2) activation to AMP-indolepyruvic acid by TdiA (adenylation domain), whose nonribosomal code points to an arylic acid rather than to amino acid-activating function [22]; (3) dimerization of two activated indolepyruvic acid monomers to the core quinone structure, which might be accomplished by the TdiA thioesterase domain, analogous to the cyclization activity of the tyrocidine thioesterase domain [23]; and, finally, (4) the possibility of oxidoreductase TdiC playing a role in reducing the keto groups of the quinone core, perhaps to prepare it for the prenyl transfer (a biosynthetic pathway is presented in Figure 5). Yet, the full metabolic pathway (e.g., at which time the two tailoring prenyl transfer reactions occur) remains elusive and will be subject to our further genetic and biochemical investigations.

These results from our *laeA*-based genome mining expedition reveal a novel, to our knowledge, genomic method to identify expressed clusters. This knowledge fills a large gap in existing technology in identifying natural products. Not only does LaeA identify actively synthesized metabolites—even an unknown compound for a given species, as demonstrated in our case—but it can do so without any structural/chemical or DNA data, requirements of traditional, e.g., PCR-based genome mining approaches. LaeA also shows no restrictions in the chemical class of metabolite that it regulates; be it polyketide, peptide, terpene, etc., the single requirement seems to be arrangement of the biosynthetic genes in a cluster [13]. This latter point is particularly promising, as the potential wealth of *Aspergillus* bioactive metabolites is enormous. Additionally, examination of several other Ascomycetes indicates a similarity in PKS number to the Aspergilli [24], thus revealing a fungal secondary metabolite capability as rich, if not richer, than evident from the two published *Streptomyces* genome projects [25, 26]. Putative *laeA* homologs exist in numerous filamentous ascomycetes genera, e.g., *Magnaporthe*, *Coccidioides*, and *Fusarium* [13]. Assuming functionality of these homologs in these and other genera, placement of the *A. nidulans* overexpression allele could be envisioned as a generic tool reaching beyond the genus *Aspergillus* to upregulate metabolite production in filamentous fungi.

Although presented as a potent mining tool in this document, it is possible that the most exciting impact of LaeA lies in deciphering the mechanism of cluster regulation and relating this mode of regulation to the ecology of the organism. Data to support models for formation, maintenance, and regulation of fungal gene clusters have been scanty [6]; an understanding of LaeA function may help resolve some of these hypotheses.

Significance

Completed fungal genome projects provide much insight into the potential metabolic capabilities and therefore accelerate the search for new natural products—be it for drug lead discovery or to help assess the risk in terms of mycotoxin production. As the number of natural product gene clusters far exceeds the number of secondary metabolites characterized from a given species, a tool is needed to identify actively transcribed gene clusters whose metabolic products may not be obvious. In this report, we describe the *Aspergillus* transcriptional regulator LaeA as this sought-after tool. As a proof of principle, we report the identification of the terrequinone A biosynthetic

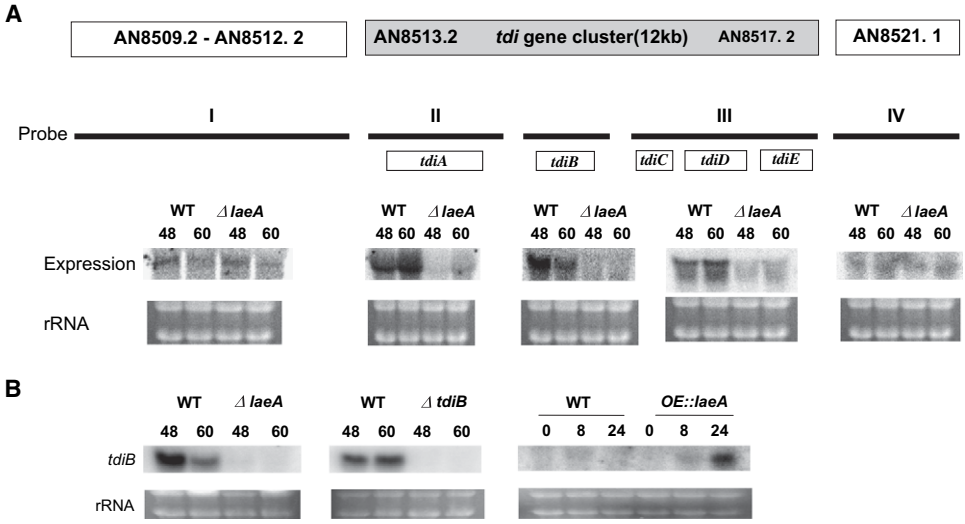


Figure 3. The *tdi* Gene Cluster

(A) Demarcation of the *tdi* gene cluster. DNA fragments covering the six putative *tdi* cluster genes (probes II and III) were expressed in wild-type, but not the *ΔlaeA* mutant. DNA fragments adjacent to the proposed cluster (probes I and IV) were expressed in both wild-type and the *ΔlaeA* mutant.

(B) *tdiB* is not expressed in a *ΔlaeA* mutant or a *ΔtdiB* mutant, but it is upregulated in the *OE::laeA* mutant.

gene cluster and predict a possible biosynthetic pathway. To our knowledge, this metabolite was unknown from *A. nidulans*, and no cluster for this entire class of compound had been reported before. In turn, the cluster could not be assigned to any known compound from *Aspergillus nidulans* before, and homologies to known natural product genes—both from streptomycetes and filamentous fungi—did not support a reliable prediction of a chemical structure. Cumulatively, the advance of our approach is that it only requires transcriptional analysis of *Aspergillus* and works even if a compound is unknown from a given species or if structural information is unavailable. Moreover, it is not limited to a particular chemical class of metabolites. As it works in other *Aspergillus* species as well, LaeA may (1) serve to explore the complete secondary metabolome of the entire genus and, (2) from the applied point of view, help develop robust producer strains, as LaeA overexpression promotes secondary metabolite production.

Experimental Procedures

Fungal Strains and Growth Conditions

Table S2 lists all fungal strains used in this study. All strains were maintained as glycerol stocks and were grown at 37ºC on glucose minimal medium (GMM), threonine minimal medium (TMM), or lactose minimal medium (LMM) [13, 27] amended with 30 mM cyclopentanone. Cyclopentanone induces *alcA(p)*, which was used to promote *laeA* expression in an overexpression (*OE::laeA*) strain. All media contained appropriate supplements to maintain auxotrophs [28].

Nucleic Acid Analysis

DNA extractions from fungal and bacterial strains, restriction enzyme digestion, gel electrophoresis, blotting, hybridization, and probe preparation were performed according to standard methods [27, 29]. Total RNA was isolated from lyophilized mycelia by using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA blots were hybridized with a 1 kb *tdiB* PCR product by primers NAlf1 and NAlr1 for the expression of *tdiB*. The *tdi* gene cluster boundary was determined by hybridizing a 6 kb PCR product (probe I by primers NCf3 and NCr3), a 4 kb PCR product (probe II by primers NCf4 and NCr4), a 4 kb PCR product (probe III by primers NCf5 and NCr5), and a 3 kb PCR product (probe IV by primers NCf6 and NCr6) to total RNA extracted from wild-type and *ΔlaeA*. Primers are listed in Table S3.

Disruption of the *tdiB* Gene

PCR technique was applied to create a *tdiA* disruption cassette in which the *tdiB* open reading frame was replaced with the *A. parasiticus pyrG* selection marker. The disruption cassette was constructed by ligating a 1.1 kb DNA fragment upstream of the *tdiB* start codon (primers NcAf1 and NcAr1, the latter with an EcoRI site) and a 1.1 kb DNA fragment downstream of the *tdiB* stop codon (primers NcAf2 and NcAr2, the latter with a HindIII site) to the EcoRI and HindIII side of the *A. parasiticus pyrG* marker gene, respectively, obtained from pBZ5 [30]. A total of 3 μl of the ligation mixture was used to amplify the resultant 5 kb disruption cassette by using the Triple master PCR kit (Eppendorf, Westbury, NY). A total of 20 μl of the PCR product was purified with a G-50 column (Pharmacia) and was then used for the disruption of the *tdiB* gene. Primers are listed in Table S3. *Pfu*Ultra (Stratagene) was used for the PCR reactions of the 5′ and 3′ flanking regions of the cassette. Strain RLMH37 was transformed by the PCR fragment. Fungal transformation essentially followed the method described by Shimizu and Keller [27], with the modification of embedding the protoplasts in top agar (0.75%) rather than spreading them by a glass rod on solid media. Five out of 27 transformants were confirmed by Southern hybridization to contain a *tdiB* gene replacement (data not shown). One of the disruptants, TJW65.7, was used for subsequent experiments.

Microarray Analysis

Arrays were generated by Nimblegen, Inc. (Madison, WI) for each annotated gene in the *A. nidulans* genome database (Broad Institute). Each gene was represented by 10 oligonucleotide probe pairs (24 bases each) consisting of a "perfect match" probe identical to a genomic sequence and a "mismatch probe" designed to differ at two positions relative to the perfect match probe. Total RNA of wild-type and *ΔlaeA* strains was prepared in duplicate from FGSC 26 (*biA1; veA1*) and RJW40.7 (*biA1; methG1; ΔlaeA::metG;veA1*) grown for 48 hr in GMM by using TRIzol reagent (Invitrogen, Carlsbad, CA), followed by RNeasy clean up (Qiagen Inc., Valencia, CA). Total RNA of wild-type and *OE::laeA* strains was prepared in triplicate from

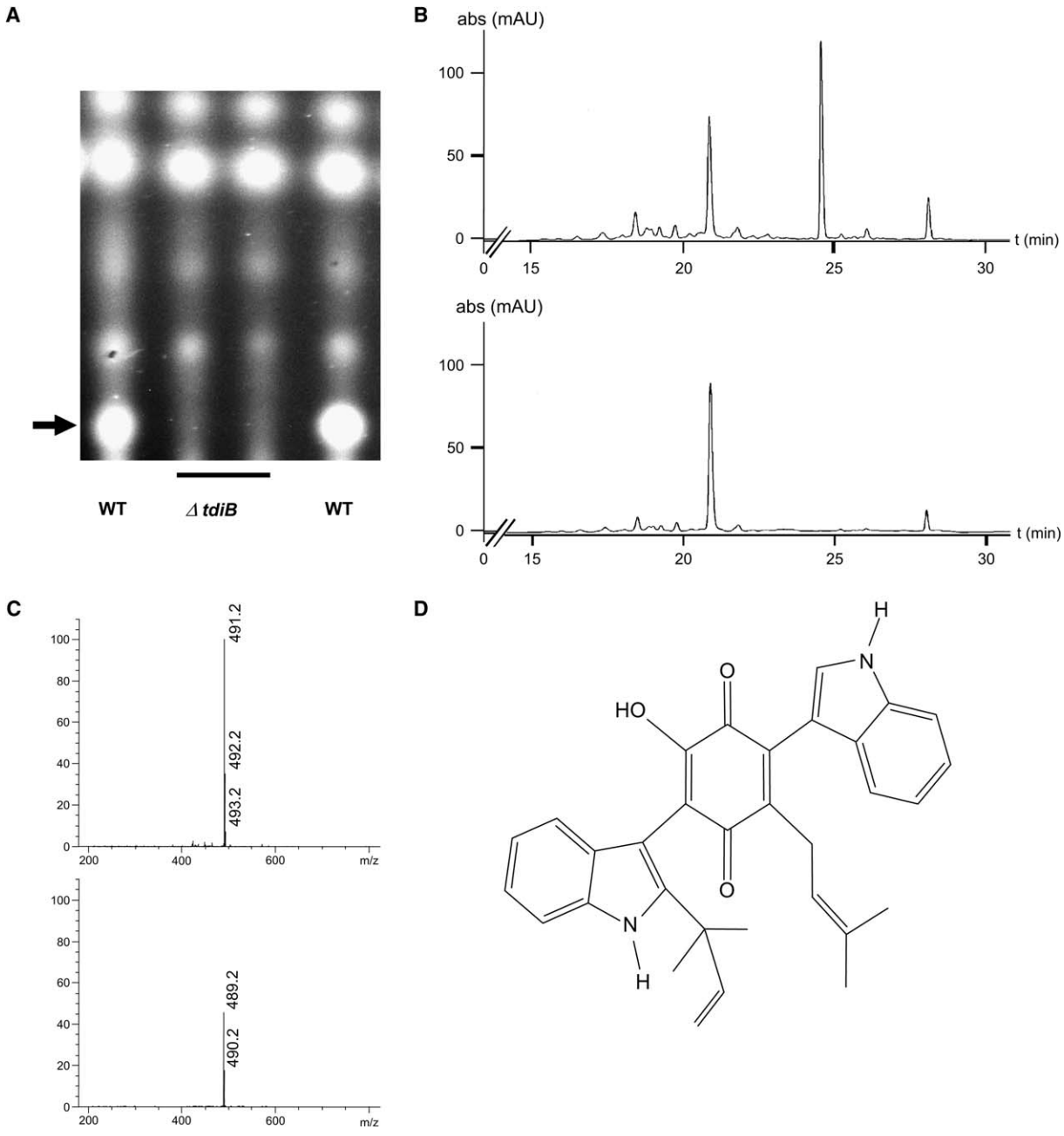


Figure 4. Lack of Metabolite Production in the *ΔtdiB* Mutant

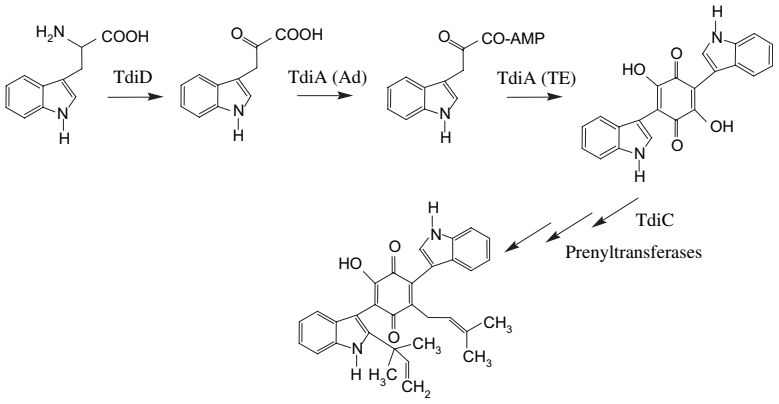
(A) Chloroform extracts from wild-type and the *ΔtdiA* mutant run on a thin layer chromatography plate in a hexane:ethyl acetate (4:1) solvent system. The arrow points at the Rf of the missing metabolite in the *ΔtdiA* mutant.

(B) Chloroform extracts from wild-type (upper panel) and the *ΔtdiB* mutant (lower panel) analyzed by HPLC. For wild-type, the two major peaks are ST, eluting after 20.9 min, and terrequinone A, eluting after 24.6 min. The chromatograms were recorded at 254 nm; the vertical axis shows milliabsorption units (mAU).

(C) Mass spectroscopy in the positive (upper panel) and negative mode (lower panel). The mass spectrum of the HPLC peak at 24.6 min was extracted; signal intensities are given as relative abundance with the 491.2 signal set as 100%. Peak 491.2 corresponds to the protonated molecule, and peak 489.2 corresponds to the deprotonated molecule.

(D) The chemical structure of terrequinone A.

FGSC 26 (*biA1; veA1*) and RJW44.2 (*biA1; methG1; alcA(p)::laeA::trpC, veA1; ΔlaeA::methG*) grown for 24 hr in LMM with 30 mM cyclopentanone (ICN Biochemicals INC, Aurora, OH) after 24 hr in GMM by using TRIzol reagent (Invitrogen, Carlsbad, CA), followed by RNeasy clean up (Qiagen Inc., Valencia, CA). Total RNA was spiked with control RNA transcripts, converted to biotinylated cRNA, and fragmented by following the Affymetrix Expression Analysis Technical Manual. Hybridization mixtures were prepared according to the array manufacturer's standard protocol by using 10 μg biotinylated cRNA, and they were incubated with the arrays overnight at 45ºC. Chips were washed, stained with streptavidin-linked Cy3 dye, and dried according to the manufacturer's protocol. Chips were scanned by using a GenePix scanner (Axon Instruments, Union City, CA). The data were imported into a Microsoft Access database, and mismatch



Terrequinone A

Figure 5. Terrequinone A Biosynthesis

Hypothetical order of the key steps: deamination (TdiD), adenylation and dimerization (TdiA, Ad = adenylation domain, TE = thioesterase/cyclase domain), and reduction (TdiC). Prenyl transfers might occur separately and at earlier points in the biosynthetic route.

probe signals were subtracted from perfect match signals and averaged across genes. These average signal values were normalized by multiplying every signal value by a scaling factor calculated as 1000 signal units divided by the average signal for the RNA spike controls. For the purpose of calculating ratios, a value of 5 signal units was substituted for genes with negative signal values (where mismatch probe signals exceeded perfect match signals). Genes dependent on LaeA for expression were determined by using EBarrays software [31, 32] to identify genes with statistically different signals between mutant and wild-type (*ΔlaeA* mutant strain to wild-type or *OE::laeA* to wild-type). Once LaeA-regulated genes had been identified, they were assigned gene identification numbers according to Broad Institute nomenclature for *Aspergillus nidulans* to verify clustered localization by using the publicly accessible genomic sequence (at http://www.broad.mit.edu/annotation/fungi/aspergillus/). Next, BLAST searches (through the Broad Institute) were carried out to check for known homologous genes in other organisms.

Chemical Analysis of the *ΔtdiB* Mutant

The *A. nidulans* TJW65.7 and *A. nidulans* wild-type were grown in 100 ml liquid GMM. The cultures were fermented at 37ºC and 200 rpm for 3 days. Upon harvest, the fermentation broth was centrifuged (10 min, 2,700 × g). The mycelium was extracted with 30 ml chloroform. The supernatant was extracted separately with an equal volume of chloroform. The organic layers were evaporated in vacuo, then redissolved in 300 μl methanol and subjected to High Performance Liquid Chromatography (HPLC). Because no differences were found, both extracts were pooled (for preparative purposes, a 4 liter fermentation was used, the solvent volumes were scaled up accordingly, and the broth was extracted twice). Analytical HPLC was performed on a Waters liquid chromatograph with an Xterra MS C-18 column (100 × 4.6 mm) and a C-18 guard column, maintained at 35ºC: detection at 254 nm (diode array acquisition: 220–500 nm). Solvent A: 0.5% (v/v) acetic acid in H_2O; solvent B: 0.5% (v/v) acetic acid in acetonitrile, flow rate: 0.5 ml min^{-1}. The gradient was: initial hold for 3 min at 20% B, then, within 23 min, at 95% B. Liquid chromatography-Mass Spectroscopy (LC/MS) in analytical and preparative scale was performed on an Agilent 1100 integrated system equipped with a Zorbax Eclipse XDB C-8 column (150 × 4.6 mm, 5 μm particle size) and C-8 guard column, essentially applying the conditions described for HPLC, using atmospheric pressure chemical ionization (APCI), and switching between positive and negative mode. For preparative HPLC, a Zorbax SB C-18 column (150 × 9.4 mm) and a C-18 guard column were used; flow rate was 3.5 ml min^{-1}. Thin layer chromatography was carried out on silica gel 60 plates with hexane:ethyl acetate (4:1, v/v) as the mobile phase. For NMR experiments, the pure compound was dissolved in acetone-d_6.

Supplemental Data

Supplemental Data including tables of fungal strains, primers, and NMR data and a figure of an LaeA-regulated gene cluster are available at http://www.chembiol.com/cgi/content/full/13/1/31/DC1/.

Acknowledgments

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