FOOD SAFETY

©2017 Food Safety Commission, Cabinet Office, Government of Japan
doi: 10.14252/foodsafetyfscj.2017024
Food Safety 2017; Vol. 6, No. 1, 7–32

Review

Aspergillus flavus Secondary Metabolites: More than Just Aflatoxins

Jeffrey W. Cary[1], Matthew K. Gilbert[1], Matthew D. Lebar[1], Rajtilak Majumdar[1], Ana M. Calvo[2]

[1]Southern Regional Research Center, ARS-USDA, New Orleans, LA, 70124, USA
[2]Department of Biological Sciences, Northern Illinois University, Dekalb, IL 60115, USA

Aspergillus flavus is best known for producing the family of potent carcinogenic secondary metabolites known as aflatoxins. However, this opportunistic plant and animal pathogen also produces numerous other secondary metabolites, many of which have also been shown to be toxic. While about forty of these secondary metabolites have been identified from *A. flavus* cultures, analysis of the genome has predicted the existence of at least 56 secondary metabolite gene clusters. Many of these gene clusters are not expressed during growth of the fungus on standard laboratory media. This presents researchers with a major challenge of devising novel strategies to manipulate the fungus and its genome so as to activate secondary metabolite gene expression and allow identification of associated cluster metabolites. In this review, we discuss the genetic, biochemical and bioinformatic methods that are being used to identify previously uncharacterized secondary metabolite gene clusters and their associated metabolites. It is important to identify as many of these compounds as possible to determine their bioactivity with respect to fungal development, survival, virulence and especially with respect to any potential synergistic toxic effects with aflatoxin.

Key words: *Aspergillus flavus*, gene cluster, mycotoxins, secondary metabolite

1. General Introduction

Chemical analysis of fungal developmental structures such as hyphae, conidia, fruiting bodies and sclerotia has uncovered a plethora of secondary metabolites, many of which are secreted into the environment[1)]. Secondary metabolites (SMs) are low molecular weight natural products that often display bioactive properties and are produced from precursors derived from primary metabolism. Secondary metabolites are, by definition, not essential to the producing organism

Received: 11 December 2017; Accepted: 9 March 2018; Published online: 30 March 2018
Corresponding author: Jeffrey W. Cary, Ph.D. Southern Regional Research Center, ARS-USDA, New Orleans, LA, USA (jeff.cary@ars.usda.gov)
The contents of this article reflect solely the view of the author(s).
This paper was presented at the 12th International Symposium "Toxins, Pathogens, and Foods: Challenges and Opportunities for Public Health" organized by the Toxic Microorganisms Panel of the United States-Japan Cooperative Program on Development & Utilization of Natural Resources.
Abbreviations: ACP: acyl carrier protein, AFs: aflatoxins, AFB_1: aflatoxin B_1, AFB_2: aflatoxin B_2, antiSMASH: antibiotics and secondary metabolite analysis shell, AT: acyltransferase, BLAST: Basic Local Alignment Search Tool, CASSIS: Cluster Assignment by Islands of Sites, CRISPR: clustered regularly interspaced short palindromic repeats, CPA: cyclopiazonic acid, DAD: diode-array detector, DANS: differential analysis by 2D-NMR spectroscopy, DH: dehydratase, DMATS: dimethylallyltryptophan synthases, DTP: ditryptophenaline, ER: enoyl reductase, GGI: 3-geranylgeranyl indole, GGPP: geranylgeranyl diphosphate, GGT: geranylgeranyl transferase, GO: gene ontology, HAD: hetero-Diels–Alder, HMM: hidden Markov Model, HPLC: high-performance liquid chromatography, HRMS: high resolution mass spectrometry, IDT: indole-diterpene, IDTC: indole-diterpene cyclase, KEGG: Kyoto Encyclopedia of Genes, KA: kojic acid, KR: ketoreductase, KS: ketosynthase, LC-HRMS: liquid chromatography and high resolution mass spectrometry, LC-MS: liquid chromatography-mass spectrometry, LIC: leporin B-iron chelate, MiBIG: Minimum Information about a Biosynthetic Gene cluster, MIDDAS-M: *motif-independent de novo detection* of *secondary metabolite gene clusters,* MIPS-CG: motif-independent prediction without core genes, MS: mass spectrometer, NMR: nuclear magnetic resonance, NRPS: nonribosomal peptide synthetase, OE: overexpression, PKS: polyketide synthase, PSI-BLAST: Position Specific Iterated-BLAST, RiPPs: ribosomally-synthesized and post-translationally modified peptides, RNA-Seq: ribonucleic acid sequencing, SAM: S-adenosyl-L-methionine, siRNA: small interfering RNA, SMs: secondary metabolites, SMURF: secondary metabolite unique regions finder, TE: thioesterase, TLC: thin-layer chromatography, UHPLC: ultra-high-performance liquid chromatography, YAIG: Tyr-Ala-Ile-Gly

and for the most part are characterized based on their impact on human and animal health. Fungal SMs are quite diverse in their chemistry and their bioactivities, being sources of beneficial therapeutic compounds such as penicillins and lovastatin as well as harmful toxins such as aflatoxins and fumonisins. The evolutionary advantages of the production, sequestration and secretion of bioactive SMs by fungi has been the subject of decades of scientific speculation. Though not essential for growth like primary metabolites, SMs do impart advantages to the producing organism. Fungi are in a constant battle with both predator and competitor organisms that they must be able to coexist, compete and interact with in their particular environmental niche. These niches, be they the human body or an agricultural field are dynamic environments subject to change where fungi must be able to adapt in order to compete for available nutrients allowing them to reproduce and disseminate thus ensuring survival. Fungi have evolved the capability to produce a wide array of SMs in response to their environments many of which play important biological roles such as virulence factors, chemical defense agents, insect attractants, protection from abiotic stressors, developmental regulators, and as chemical signals for communication with other organisms.

Aspergillus flavus is a saprophyte in soils worldwide where it survives by obtaining nutrients from dead or decaying organic matter. In soils, *A. flavus* can survive as propagules in the form of as asexual conidia or overwintering structures termed sclerotia that can germinate when environmental conditions become conducive to growth to form hyphae and conidiophores. With the discovery of a sexual stage, *A. flavus* sclerotia were shown to function as fruiting bodies, termed stromata, that harbor the sexual ascospores[2]. *A. flavus* is a frequent contaminant of agricultural crops such as maize, peanut, cottonseed, and a number of tree nuts, both pre- and post-harvest, where it produces the toxic and carcinogenic group of SMs known as aflatoxins (AFs)[3]. Contamination of susceptible crops with AFs poses serious health risks in developing countries and significant economic losses in the U.S. and other developed countries[4,5]. AF contamination of crops usually results from opportunistic invasion by the fungus due, in most cases, to mechanical or insect damage of the plant cell wall[6].

The *A. flavus* genome is estimated to be approximately 37 Mb and capable of encoding about 13,000 predicted functional genes[7]. Though best known for its production of AFs, *in silico* analysis of the *A. flavus* 3357 genome sequence utilizing the web-based **S**econdary **M**etabolite **U**nique **R**egions **F**inder (SMURF) algorithm (http://www.jcvi.org/smurf) has shown that it has the potential to produce numerous other SMs. Using SMURF, Georgianna et al.[8] predicted the genome of *A. flavus* to contain 55 SM gene clusters. The total number of SM clusters in *A. flavus* can be increased to 56 when including the kojic acid (KA) gene cluster identified originally in *A. oryzae* by Marui et al.[9]. However this may be an underestimation, as other SM gene cluster prediction programs have identified even greater numbers of gene clusters in *A. flavus*[10]. Although about 40 SMs have been identified from *A. flavus* cultures[11–13] (**Table 1**), metabolites have only been experimentally assigned to twelve of the predicted 56 *A. flavus* SM clusters (see section 5). Elucidation of the products of the remaining SM gene clusters is confounded by the fact that many are transcriptionally silent, inactive due to gene mutations or expressed at extremely low levels thus making identification of their respective products problematic under laboratory growth conditions. These types of gene clusters are termed cryptic or orphan and identification of their metabolite(s) cannot be achieved using classical knockout of a cluster gene followed by comparative metabolic analysis of the mutant and the wild-type by liquid chromatography-mass spectrometry (LC-MS). Analysis of ribonucleic acid sequencing (RNA-Seq) data from a number of *A. flavus* strains growing on artificial media indicates that approximately half of the gene clusters are silent or expressed at very low levels[18]. A number of methods have been used to overcome silent gene clusters thereby enabling identification of some previously unknown cluster metabolites[19–21]. Determination of the chemical structure and biological activities of metabolites produced by these uncharacterized gene clusters will undoubtedly provide invaluable insights into the ecology of *A. flavus* as well as other fungal plant pathogens harboring similar gene clusters. It is important to identify as many of these SMs as possible to determine their bioactivity with respect to fungal development, survival, virulence and especially with respect to any potential synergistic toxic effects with AF.

In this review, we present information on the identification and characterization of SM gene clusters and their concomitant metabolites from *A. flavus*. A number of gene clusters in *A. flavus* are highly homologous to gene clusters from other filamentous fungi for which the associated metabolite has been identified. However, this review will only discuss those SM gene clusters for which the attendant metabolite(s) have been experimentally confirmed in *A. flavus*. Information will be presented on the genomic, biochemical, bioinformatic and molecular methods used to elucidate the genes and their encoded enzymes/proteins that are responsible for biosynthesis of SMs in *A. flavus*. Due to the many comprehensive reports available on AFs[3,22,23] and cyclopiazonic acid (CPA)[24,25], these two SMs will not be covered in this review. Instead, we will focus on recently characterized *A. flavus* SM gene

doi: 10.14252/foodsafetyfscj.2017024

Table 1. *A. flavus* secondary metabolites

Compound	Class	Reference
3-nitropropionic acid	propionate	12
4-hydroxymethyl-5-hydroxy-2H-pyran-2-one	pyranone	13
aflatoxins	difurocoumarolactone	12
aflatrem	indole diterpene	12
aflavarin	bicoumarin	13
aflavazole	indole diterpene	12
aflavinines	indole diterpene	12
asparasone	anthraquinone	13
asperflavin	anthrone	12
aspergillic acids	pyrazinone-hydroxamic acid	12
asperipin-2a	cyclic peptide	14
aspirochlorines	epithiodiketopiperazine	13
cladosporin	isocoumarin	12
cyclo(D-N-methyl-Leu-L-Trp)	diketopiperazine	13
cyclopiazonic acid	indole-tetramic acid	12
ditryptophenaline	diketopiperazine	12
ferriaspergillin	pyrazinone-hydroxamic acid	12
flavoglaucin	quinol	13
flufuran	furan	13
gliotoxin	epithiodiketopiperazine	13
indole	indole	13
isoflavipucine	pyridone	12
itaconic acid	succinate	13
kojic acid	pyranone	13
kotanin	bicoumarin	13
leporins	pyridone	15
lna and *lnb* piperazines	piperazine	16
lovastatin	nonaketide	13
mellein	isocoumarin	13
miyakamides	didehydrotryptamine-dipeptide	13
oxylipins	fatty acid	13
parasiticol	difurocoumarolactone	13
parasiticolide A	terpene	13
paspaline	indole diterpene	12
paspalinine	indole diterpene	12
penicillin	beta-lactam	13
sterigmatocystins	difuroxanthone	13
ustiloxin B	cyclic peptide	17

clusters and their metabolites. In addition to discussion of the genetics and chemistry of *A. flavus* SMs and their gene clusters, the potential roles these metabolites play in the biology of the fungus will also be presented.

2. Functional Genomics and Secondary Metabolism

Research approaches in fungi utilizing functional genomics may entail a range of molecular, biochemical or bioinformatic tools, however, most often the term describes research aimed at determining gene or protein function and interaction utilizing a genome-wide analysis of data. Such experiments include a bottom-up approach where specific genes are targeted for disruption or ectopic expression and the resulting effect on transcriptional regulation or biological processes is determined[26–29]. Other common approaches include the use of chemical treatment, environmental conditioning, host-pathogen interaction or other stimuli relevant to the investigation to identify novel genes, proteins, interactions, expression profiles, and entire processes responding to the stimuli[30–33]. Alternatively, functional genomics can utilize genome-wide information to elucidate gene function with techniques such as such as gain-of-function cDNA screens[34], small interfering RNA (siRNA)-based knockdown genome libraries[35], or clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 approaches designed to target a majority of the transcriptome[36]. In addition, proteomics and metabolomics have incorporated functional genomics as a component of large scale screening approaches, such as mass-spec analysis of 2D protein gels combined with genome data[37,38], high-throughput yeast two hybrid interaction assays[39], carbon flux algorithms correlated with gene expression data[40] and transcriptome-metabolome co-expression analysis[41].

Through the use of knockout technologies, microarray analysis, RNA-sequencing, and *in silico* analysis, SM cluster prediction is continually evolving to become more targeted and efficient in its predictive power. Section 2 of this review will focus on the functional genomic tools using genome-wide expression analysis to elucidate secondary metabolic gene cluster activation and regulation in *A. flavus*.

2-1. Microarray Technology

Microarray technology was among early approaches characterizing *A. flavus* gene function[42], natural antisense transcripts[43], and secondary metabolism, with an initial focus on AF regulation[44–48]. Subsequent to this, the examination of other SM gene clusters using customized microarrays were reported[8,29,49,50]. An early effort at this was by Georgianna et al.[8] whereby a collection of microarray data from 28 different treatments was used to comprehensively associate cluster expression patterns depending on treatment and environmental conditions. Here four clades, or expression patterns, of the 55 SMURF-identified clusters was described, with about half of the clusters determined to be low or non-expressing at all conditions analyzed. Another clade was characterized by high expression at most conditions and included clusters 23 (leporins), 55 (CPA), 26 (unknown) and 36 (unknown). Cluster 26 was also identified by Ehrlich and Mack[18] and Wang et al.[51] as differentially expressed on solid media and during pathogen-host interaction in peanut, respectively. The study by Georgianna et al.[8] was limited in that it could not account for the effect of accessory enzymes, but provided one of the first comprehensive profiling of secondary metabolic genes in *A. flavus*.

In 2013, Reverberi et al.[49] examined 24 SMURF-identified gene clusters that were described to be up or down regulated in two separate comparisons. In the first, *A. flavus* grown in liquid media with and without autoclaved maize kernels indicated up regulation of what is now known to be the gene clusters producing a pyrazinone (cluster 11), aflatrem (cluster 15), leporins (cluster 23), and down regulation of the AF (cluster 54) cluster. A second comparison whereby *A. flavus* was grown on autoclaved kernels and live kernels in the field identified effects of 16 different clusters, including the pyrazinone, leporin, CPA and AF clusters. Cary et al. observed the altered co-expression of clustered genes using microarray analysis of a *veA* knockout strain to identify the sclerotia-related clusters that synthesize aflavarin[29] and asparasone[52]. Both aflavarin (cluster 39) and asparasone (cluster 27) were among the clusters predicted to be sclerotia-related in the Georgianna et al.[8] microarray analysis and by Ehrlich and Mack[18] based on the temporal correlation of sclerotia development and cluster expression.

2-2. RNA Sequencing

RNA-sequencing technologies are quickly replacing most other means of high-throughput transcriptomics, given their decreasing cost, high accuracy, and the increased availability of bioinformatic services[53–55]. Ehrlich and Mack[18] was among the first to utilize RNA-sequencing to characterize secondary metabolic gene clusters in *A. flavus*, primarily for annotation purposes in identifying absolute expression activity and defining cluster boundaries. The authors identified eight clusters demonstrating high expression levels under their conditions and eleven clusters correlating with sclerotial development. In addition to asparasone and aflavarin mentioned previously, the cluster for leporin production was also among those predicted[15].

To date there has not been numerous use of RNA-seq combined with gene knockout technology in *A. flavus*, however RNA-seq was conducted on the small sclerotia producing *A. flavus* strain AF70 to examine the effects of knocking out the zinc-finger transcription factor *nsdC*. Inactivation of *nsdC* resulted in decreased aflatrem production, as well as decreased expression of genes encoding the backbone enzymes for penicillin, asparasone, ustiloxin B, and AF biosynthesis[26]. Clusters 5, 20, 41, 44, and others also showed altered transcriptional profiles in the *nsdC* knockout strain. Metabolites produced by these clusters remain to be identified.

In addition to gene knockout strategies, chemical treatment and the manipulation of environmental conditions combined with RNA-seq technology has successfully characterized various gene clusters in *A. flavus*. Treatment of *A. flavus* with resveratrol, a naturally occurring phytoalexin and antifungal compound, affected 17 genes in 13 clusters, 2 of which were nonribosomal peptide synthetases (NRPSs) or NRPS-like enzymes (cluster 3 and 45, respectively)[56]. Although fungi are thought to not undergo DNA methylation[57] the DNA methyltransferase inhibitor 5-azacytidine has been speculated to interfere with toxisome formation[50]. Upon treatment of *A. flavus* with 5-azacytidine, RNA-seq revealed an increase in expression of several genes of cluster 27 (asparasone), cluster 35 (piperazine), cluster 55 (CPA) and moderately decreased expression of AF cluster genes[58]. In another RNA-seq study, the plant volatile, decanal, impacted expression of genes present in 26 of the 55 clusters[59]. Finally, because drought-stress leads to increases in reactive oxygen species, a RNA-seq study examined the effects of oxidative stress (exposure to H_2O_2) on 3 strains of *A. flavus* synthesizing varying levels of AF. The focus of this study on secondary metabolic gene products besides AF was limited, however the authors did indicate genes in the aflatrem and KA gene clusters were affected[60].

Recently the use of functional genomics, and in particular RNA-sequencing, to measure the genome-wide response to changing environmental conditions has become more widespread[30,61], and whereas early experiments focused on the AF gene cluster, secondary metabolic cluster prediction and transcriptome-wide profiling has allowed for more complete analysis. The influence of temperature or water activity combined with RNA-seq has been used to examine small non-coding RNAs[62], development[63] and SM production[64,65]. Yu et al.[65] showed 10 of the 55 gene clusters had higher expression at 30°C as compared to 37°C (cluster 1, 10, 11, 19, 20, 21, 24, 45, 54, and 55) and cluster 3 decreasing at lower temperatures. Medina et al.[64] found genome-wide, a decreased transcriptome response to changing environmental conditions correlating with low water activity or high temperature, with water being the primary predictor. As an example, the CPA cluster (cluster 55), was lower in every condition examined due to low water availability, regardless of temperature conditions. Preliminary or small-scale studies have indicated that other environmental conditions, for example, those related to climate change also impact secondary metabolism[61,66,67]. Studies are ongoing to examine this impact on a genome-wide scale.

2-3. Bioinformatic Tools Used for Cluster Prediction in *A. flavus*

The most commonly used bioinformatic tools for RNA-seq analysis such as KEGG (Kyoto Encyclopedia of Genes)[68], gene co-expression network analysis[69] and gene ontology (GO) enrichment analysis[70] have the potential to facilitate the analysis of large data sets and have been used in functional genomics analysis of *A. flavus*[26,52,56,58]. While these methods will categorize genes, determine categories significantly enriched, and identify similar gene expression patterns, the identification of cluster function is generally beyond these tools. To facilitate cluster prediction in *A. flavus* multiple computational approaches have been developed[71] (**Table 2**). During development and testing, *A. flavus* clusters were used as test sequences for the motif-dependent program SMURF, and the motif-independent programs: **M**otif-**I**ndependent **D**e Novo **D**etection of **S**econdary **M**etabolite Gene Clusters (MIDDAS-M), and **M**otif-**I**ndependent **P**rediction without **C**ore **G**enes (MIPS-CG). Among the more popular programs, SMURF, developed by Khaldi et al.[72] at the J. Craig Venter Institute, uses hidden Markov Model (HMM) searches to predict the presence of TIGRFAM and Pfam conserved protein domains associated with various SM backbone enzyme genes. These include NRPSs, polyketide synthases (PKSs), hybrid NRPS-PKS enzymes and prenyltransferase enzymes (DMATS, dimethylallyltryptophan synthases). SMURF predictions are limited in the type of cluster predicted, but are generally regarded as consistent with other forms of prediction[73,74]. While SMURF has demonstrated significant effectiveness as evidenced by the publications it has contributed to, the last update was April 2015 and technical support to resolve discrepancies appears unavailable[75,76].

Alternatively, the popular algorithm **anti**biotics and **S**econdary **M**etabolite **A**nalysis **Sh**ell (antiSMASH), is subject to frequent upgrades and user input, with versions made available as recently as April 2017[77–79]. The software serves as a pipeline that integrates multiple published analysis types to predict a wide range of cluster types and enzyme domains, including PKS/NRPS's, terpenes, indoles, siderophores,

Table 2. Comparison of categories of *A. flavus* predicted secondary metabolite gene clusters using different web-based computational programs.

Category	SMURF[a]	antiSMASH[b]	MIDDAS-M[c]
PKS (Type I and III)	25	20	15
NRPS	18	11	12
PKS-NRPS hybrid	2	3	3
DMAT/Terpenes[d]	8	13	7
NRPS-Like	14	–	20
PKS-Like	3	2	–
Indole[e]	–	5	4
RiPS	–	–	1[f]

[a]Pre-computed data from www.jcvi.org/smurf. Differences from Georgianna et al. (2010)[8] data could be contributed to changes in the SMURF algorithm or sequences used.
[b]Obtained by running antiSMASH v. 4.0.0 (www.fungismash.secondarymetabolites.org) using *A. flavus* NRRL 3357 genome sequence (EQ963472).
[c]Supplementary data obtained from Umemura et al. (2013)[10].
[d]antiSMASH categorizes DMAT's as terpenes, while SMURF defines them independently.
[e]Indole clusters in antiSMASH determined by hidden Markov modeling using the StaD-like chromopyrrolic acid synthase domain (Medema et al. 2011)[78].
[f]MIDDAS-M identified the ustiloxin B cluster. *A. flavus* genome analysis has identified an additional 7 potential RiPS (Nagano et al. (2016)[14].

and in recent versions, Ribosomally-synthesized and Post-translationally modified Peptides (RiPPs). At the core of antiSMASH, like SMURF, amino acid sequence translations are identified as containing signature domains using hidden Markov Models, and regions up to 20kb away are searched for accessory enzymes. Other programs within antiSMASH utilize trained and phylogentic-based algorithms to predict substrate structure based on motifs within acyltransferase or adenylation domains, from which a final structure is derived based on other domains present. In early versions the "greedy" approach of boundary prediction led to excessively large clusters being predicted, however recent versions have attempted to optimize this element by incorporating sequence-based algorithims (CASSIS and ClusterFinder) that use known regulatory motifs and protein domains to assist in border prediction. Additionally, antiSMASH incorporates data from the Minimum Information about a Biosynthetic Gene cluster (MiBIG) database which attempts to incorporate experimentally-determined SM data.

Finally, MIDDAS-M does not utilize characterized motifs as a basis for prediction[10]. Rather, it utilizes both genomic and transcriptomic data to calculate expression ratios of neighboring genes, and large induction ratios to indicate cluster activity. This is beneficial when attempting to identify novel clusters that do not contain motifs searched for by other programs. The authors claim that even SM biosynthetic genes not co-localized, such as those synthesizing KA in *A. oryzae* can be detected[80]. The ustiloxin B biosynthetic gene cluster is unique in that the peptide is ribosomally synthesized. The ustiloxin B cluster was detected by MIDDAS-M, which was later experimentally-validated[17]. A comprehensive comparison of MIDDAS, antiSMASH and SMURF using *A. flavus* was conducted by Umemura et al[80]. We have not found other software programs utilized extensively for cluster predictions in *A. flavus*. In our own experience, this is sometimes due to difficulty of use, inaccurate cluster predictions, a failure to maintain basic but essential updates beyond publication, or technical defects (bugs) with no technical support.

2-4. Cluster Prediction in the Age of Genomics

With the recent increase in large SM and genomic data sets and databases such as ClusterMine360, IMG-ABC, and MIBiG, strategies using comparative and computational genomics and transcriptomics for cluster prediction are becoming more effective[73,74,81]. These approaches must go beyond using genes in other species of *Aspergillus* to predict *A. flavus* SM gene clusters, primarily because the degree of homology needed and role of accessory proteins and "hypotheticals" is unknown. Certainly more than backbone gene identity is important. For example, PKS enzymes often share high degrees of similarity with unrelated PKSs. The PKS-encoding gene *afvB* (AFLA_108550) in *A. flavus* shares identity (E value =0, 43.8% aa identity) to the *A. nidulans* PKS encoded by *mdpG* (AN0150), however the *A. nidulans* product monodictyphenone is structurally dissimilar to

aflavarin[82], and none of the aflavarin cluster accessory enzymes are thought to be located near *mdpG*. Thus **B**asic **L**ocal **A**lignment **S**earch **T**ool (BLAST) or bioinformatic approaches must involve a multigene approach.

In *A. flavus*, one example of such an approach is the discovery that ustiloxin B is ribosomally synthesized[17] and that two genes in the cluster, *ustYa/Yb*, are found in 19 loci in the *A. flavus* genome, 9 of which are associated with a ustiloxin-like precursor sequence (ust-RiPS)[14]. In the case of one of these loci, knockout of the *ustYa/Yb* genes led to the identification of the novel compound, asperipin-2a[14]. The authors here developed a pipeline utilizing PSI-BLAST, which iteratively refines searches based on conserved amino acids, signal peptide detection software, and BLASTClust to further sort precursor proteins. Another method utilized in *A. flavus* by our group involves using known eukaryotic clusters in the MIBiG database[83] as queries against sequenced strains of *A. flavus*. The software program multigeneblast[84] will search a genome or set of sequences using multiple sequences as the query, thus allowing for gene rearrangement and differing degrees of identity. Hits can then be associated with the known metabolite from the database. Using this approach we can putatively identify several uncharacterized clusters in *A. flavus*. For example, all 22 of the genes in the experimentally verified *A. oryzae* aspirochlorine cluster are present as a cluster in *A. flavus* (cluster 21). Similar results are obtained for tenellin, chaetoglobosin, and trans-resorcyclide with varying levels of identity. As databases such as this increase in user contribution, predictive powers of such tools is likewise expected to increase.

3. Regulation of Secondary Metabolism in *A. flavus*

3-1. Global Regulation

The genetic mechanisms that regulate secondary metabolism in fungi have been the subject of intense investigation[21,85]. In this section, we focus on the regulation of SM production in *A. flavus* along with recently published literature that has not been presented in previous review articles. The role of LaeA, a putative histone methyltransferase, as a global regulator of development and SM production in *A. flavus* as well as other *Aspergillus* species, has been the subject of several reports[86,87]. Deletion of *laeA* in *A. flavus* resulted in complete loss of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), and CPA production, whereas, over-expression of *laeA* did not have a significant effect on production of these SMs[86]. The presence of other SMs including paspaline, paspalinine, aflatrem, and aflavinines (see section 5–5) were difficult to detect in wild-type *A. flavus*, however, in the *A. flavus laeA* over-expressor strain, levels were increased such that they could be readily detected[86]. Kojic acid (KA) production was lost in *A. flavus* Δ*laeA* mutants and levels were not increased due to over-expression of *laeA*. Kojic acid production was also abrogated in an *A. oryzae* Δ*laeA* mutant due to down-regulation of KA biosynthesis genes[88]. In addition, *laeA* has also been shown to regulate the *A. flavus* lna and lnb gene clusters (see section 5–4) involved in the production of piperazine and morpholine SMs[16].

The light-responsive protein, VeA is a component of a heterotrimeric, nuclear-localized protein complex, designated the velvet complex, which also includes LaeA and the velvet-like protein VelB. The velvet complex is responsible for regulation of development and SM production in numerous filamentous fungi and was shown to positively regulate the production of AF, CPA, and aflatrem in *A. flavus*[89,90]. Production of CPA was reduced by 48–66% in the Δ*veA* mutant (vs. control) depending upon growth in light or dark conditions. An *A. flavus* Δ*velB* mutant was unable to produce sclerotia and AFs[28]. A transcriptomic study in *A. flavus* showed that 28 SM gene clusters contain at least one gene whose expression is differentially regulated by the presence or absence of *veA*[29]. Characterization of the aflavarin gene cluster (see section 5–3) showed that *veA* directly regulates the production of aflavarin with all of the cluster genes significantly down-regulated in a Δ*veA* mutant, demonstrating direct regulation of this cluster by VeA. Functional characterization of the asparasone A gene cluster (see section 5–1) in *A. flavus* showed a significant down-regulation of the polyketide synthase gene (*pks27*) and the Zn(2)-Cys(6)-type transcription factor gene *znf27* in a Δ*veA* mutant resulting in loss of asparasone production[52].

Another global regulatory gene, *nsdC*, mainly known for its role in development in *A. nidulans*[91], was also shown to be required for AF production in *A. flavus*[27]. In addition to loss of AF biosynthesis, *A. flavus* Δ*nsdC* mutants also demonstrated reduced aflatrem content compared to a control strain. RNA-seq analysis of the Δ*nsdC* mutant revealed differential expression of genes associated with development and production of SMs such as AFs, penicillin, and aflatrem[26]. The studies presented here suggest a coordinated action between global regulators and cluster specific transcription factor genes in regulating SM production in *A. flavus*.

The Far transcription factor was shown to play an important role in fungal virulence and AF production through its involvement in lipid metabolism. The *A. nidulans farA* and *farB* genes were shown to regulate expression of genes associated with fatty acid catabolism[92]. Further characterization of *far* genes in *A. flavus* showed their essential roles in mitochondrial beta oxidation of fatty acids and regulation

of genes involved in unsaturated fatty acid biosynthesis[93]. Deletion mutants of *A. flavus farA* and *farB* showed aberrant growth phenotypes when grown on medium-chain fatty acids. Aflatoxin production was significantly decreased in a *ΔfarB* mutant (vs. control) both *in vitro* and during maize seed infection accompanied by reduced sporulation, suggesting a role of *farB* in lipid metabolism contributing to fungal pathogenesis and AF production. Another Zn(2)-Cys(6) transcription factor gene, *aswA*, has been demonstrated to affect sclerotial production and associated sclerotial SMs in *A. flavus*[94]. The Δ*aswA* mutant produced higher numbers of deformed, non-pigmented sclerotia and no asparasone A, aflatrem, dihydroxyaflavinine, or aflavarin could be detected from sclerotia as compared to the control strain. Aflatoxin and CPA content were not affected by the *aswA* mutation. In a recently published study, Cary et al.[95] demonstrated that a homeobox transcription factor gene, *hbx1*, is required for production of conidiophores, sclerotia, and the SMs AF, CPA, and aflatrem in *A. flavus*. Upon forced overexpression, a globally-acting C_2H_2 zinc-finger transcription factor, MtfA, was shown to be required for normal development and AF production in *A. flavus*[96].

Besides DNA-binding transcription factors, other regulatory factors with diverse functions have been found to regulate secondary metabolism in *A. flavus*. Examples include RmtA, a putative arginine methyltransferase, shown to be required for production of normal levels of AFB_1 and other uncharacterized SMs[97]. Similar regulation of SM production in *A. flavus* was also observed by RtfA, a putative RNA-Pol II transcription elongation factor, in coordination with VeA and LaeA[98]. The *ΔrtfA* mutant exhibited a significant decrease in sclerotia production along with reduction in AF and other unknown SMs.

3-2. Cluster-specific Regulation

In addition to global regulation of SM gene clusters, cluster-specific transcription factors are also involved in regulation of SM production in *A. flavus*. One of the most studied cluster specific transcriptional regulators is the *aflR* gene present in the AF gene cluster. The *aflR* gene is a DNA-binding, Gal4-type Zn(2)-Cys(6) transcriptional activator that regulates expression of most AF cluster genes[99–101]. *A. flavus aflR* deletion mutants do not make AFs due to lack of expression of cluster genes. AflR binds to the palindromic sequence 5′-TCGN5CGA-3′ present in the promoter region of most AF biosynthetic genes[102,103]. Aflatoxin biosynthesis is also regulated by *aflS (aflJ)* located adjacent to and divergently transcribed from *aflR*[104]. The exact role of *aflS* in AF biosynthesis is not clear despite intense investigation. Deletion of *aflS* in *A. flavus* did not appear to affect expression of AF cluster genes however *aflS* deletion mutants did not produce AF[104].

Inactivation of the *A. flavus* leporin cluster (see section 5–2) Zn(2)-Cys(6) transcription factor gene, *lepE,* resulted in significant down-regulation of all the cluster genes[15]. Over-expression of *lepE* produced orange-red pigmented hyphae due to the synthesis of the 2-pyridone, leporin B. The asparasone A gene cluster contains a Zn(2)-Cys(6) transcription factor gene, *znf27,* that when inactivated resulted in transformants that produced lighter, reddish-brown sclerotia compared to the dark brown sclerotia of the control strain. The reduced sclerotial pigmentation was due to lower levels of asparasone A production in the *znf27* mutant. Gene expression analysis showed a significant reduction in expression of asparasone cluster genes in the Δ*znf27* mutant compared to the control indicating that *znf27* functions as a pathway-specific transcription factor. A pathway-specific C6-type transcriptional regulator gene present in the ustiloxin B gene cluster (see section 5–6), encoded by *ustR,* was identified and its overexpression resulted in an increase of ustiloxin B production by about 5-fold while deletion abrogated ustiloxin production[14,17]. Disruption of KA gene cluster (see section 5–8) Zn(2)-Cys(6) transcriptional activator gene, *kojR*, resulted in a significant down-regulation of expression of KA biosynthetic genes *kojA* and *kojT* accompanied by a complete loss of KA production[9]. Over-expression of *kojR* resulted in >3-fold higher expression of KA biosynthetic genes along with >3-fold increase in KA production compared to the control strain demonstrating direct regulation of KA biosynthesis by KojR.

3-3. Methods to Activate Silent Secondary Metabolite Gene Clusters

In fungi and other micro-organisms, considerable interest lies in understanding the production of SMs by cryptic or silent gene clusters that only produce their associated metabolites when exposed to specific environmental conditions not normally duplicated during growth on standard laboratory media. Several methods have been used to activate silent SM gene clusters in fungi and these have been summarized in a number of excellent reviews[21,105–107]. These include over-expression of global or pathway-specific transcriptional regulators, expression of SM cluster genes in heterologous hosts, co-culture of a fungal strain with another micro-organism, proteomics, exposure to epigenetic modifiers and antibiotics and alteration of media composition. In the genus *Aspergillus*, most experiments to activate silent SM gene clusters have been conducted in the model species, *A. nidulans*, so there is a need to extend these approaches to *A. flavus*.

doi: 10.14252/foodsafetyfscj.2017024

4. Chemistry of Secondary Metabolites

4-1. Chemistry of Cluster Backbone Genes

Secondary metabolites produced by fungi exhibit a broad range of chemical diversity. Several classes of biosynthetic backbone genes give rise to a plethora of complex chemical structures that impact human health and agriculture. These backbone genes encode for enzymes, most of which are multifunctional megasynthases, that biosynthesize the metabolites' core structure. Tailoring enzymes then operate on the core structure to afford a diverse suite of SMs with varying functions, many of which have been shown to enhance the fitness of the fungi. **Table 3** provides a list of features associated with experimentally characterized secondary metabolite gene clusters in *A. flavus.*

4-1-1. Polyketide synthases (PKSs)

Fungal polyketide metabolites are biosynthesized by iterative, type I PKSs and have highly diverse chemical architecture[112–114]. A type I PKS contains multiple modular catalytic domains encoded by one gene. Three domains are necessary for polyketide biosynthesis: the acyltransferase (AT) domain binds acetyl-CoA or malonyl-CoA starter units, the ketosynthase (KS) domain catalyzes a Claisen condensation that extends the polyketide chain with additional malonyl-CoA or methylmalonyl-CoA units, while the acyl carrier protein (ACP) tethers the elongating polyketide chain to the PKS through a phosphopantetheine linkage. Iterative PKSs use the same AT and KS catalytic domains to add multiple malonate units to the growing polyketide. Additional reductive tailoring of the growing polyketide chain can occur if the PKS harbors functional ketoreductase (KR), dehydratase (DH), or enoyl reductase (ER) domains. Typically, a thioesterase (TE) domain severs the thioester-linked PKS-polyketide bond, liberating the nascent polyketide as a linear molecule or a macrolactone. After release from the PKS, the polyketide is further transformed by oxidases and oxygenases resulting in SMs with abundant structural complexity[115]. PKSs in *A. flavus* produce AFs, aflavarin, and asparasone A (see sections 5–1 and 5–3).

4-1-2. Nonribosomal peptide synthetases (NRPSs)

Fungal nonribosomal peptides are biosynthesized by NRPSs from proteinogenic and non-proteinogenic amino acids[113,116]. NRPSs contain multiple catalytic domains arranged in modules. Each module contains an adenylation (A) domain that activates a specific amino acid using ATP, a peptidyl carrier (P) domain, which utilizes a phosphopantetheine linker analogous to ACP domains in PKSs, and a condensation (C) domain that catalyzes peptide bond formation. Multiple modules are arranged in an assembly-line fashion whereby each module adds one amino acid to the growing peptide chain. Many NRPSs contain a TE termination domain that catalyzes peptide release. In some fungal NRPSs, the peptide chain is liberated from the NRPS by a condensation-like termination domain (C_T), which affords a cyclic peptide[117,118]. "NRPS-like" proteins contain A and T domains but lack C domains and terminate with a thioester reductase (R) domain that liberates the tethered amino acid or peptide as an aldehyde. NRPSs in *A. flavus* produce ditryptophenaline and NRPS-like enzymes produce several piperazine natural products (see sections 5–4 and 5–7).

4-1-3. Hybrid backbone genes

Hybrid PKS-NRPSs biosynthesize SMs the have both polyketide and amino acid components. *A. flavus* utilizes a hybrid PKS-NRPS enzyme (KS-AT-ACP-C-A-T-R) that forms an intermediate in CPA acid biosynthesis from a mevalonate and two acetate moieties using PKS domains and a tryptophan residue using NRPS domains[119]. *A. flavus* also produces leporins using a hybrid PKS-NRPS (see section 5–2).

4-1-4. Dimethylallyl tryptophan synthases (DMATs)

Fungal dimethylallyl tryptophan synthases alkylate tryptophan or other aromatic moieties with the isoprenoid substrate dimethylally pyrophospate[120]. A DMATS in *A. flavus* prenylates an intermediate in the biosynthesis of CPA[119,121]. The biosynthetic pathway of aflatrem in *A. flavus* also contains a DMATS-catalyzed prenylation (see section 5–5).

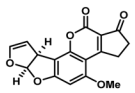
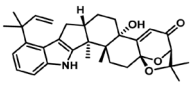
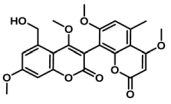
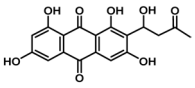
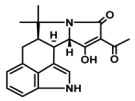
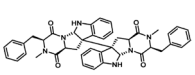
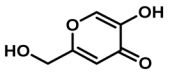
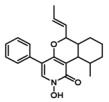
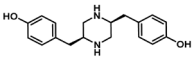
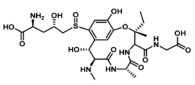
4-1-5. Meroterpenes

Meroterpenes are SMs that are partially derived from terpenoid biosynthesis pathways. Indole diterpenes are meroterpenes that contain a cyclic diterpene skeleton derived from geranylgeranyl diphosphate (GGPP) and an indole moiety derived from indole-3-glycerol phosphate[122]. Biosynthesis of these molecules is complex and involves a GGPP synthase as well as variety of oxidases. *A. flavus* produces an assortment of indole diterpenes including aflatrem and aflavinines (see section 5–5).

4-2. Methods of Analysis

Robust chromatographic techniques have been used extensively to detect mycotoxins and other SMs produced by fungi. Thin-layer chromatography (TLC) is the most accessible and, due to its simplicity and low cost, the most

Table 3. Features of *A. flavus* secondary metabolite gene clusters

Metabolite[a]	Cluster number[b]	Backbone gene classification	Cluster-specific transcriptional regulator	Number of genes in cluster[c]	Putative biological function	Representative structure	Reference
Aflatoxin	54	PKS	Yes	30	antiinsectan/oxidative stress resistance?		3,22,23
Aflatrem	15 and 32	DMATs/GGPPs	No	5 and 3	antiinsectan/ antifeedant		108,109
Aflavarin	39	PKS	No	5	antiinsectan/sclerotial development		29
Asparasone A	27	PKS	Yes	4	antiinsectan/sclerotial pigment/abiotic stress resistance		52
Cyclopiazonic acid	55	DMATs/hybrid PKS-NRPS	No	5	mammalian antifeedant?		24,25
Ditryptophenaline	4	NRPS	No	3	?		110
Kojic acid	NA	oxidoreductase	Yes	3	insect antifeedant/ antioxidant?		111
Leporin	23	hybrid PKS-NRPS	Yes	9	insect antifeedant/ sclerotial development		15
Piperazine	35 and 48	NRPS-like	No	6 and 6	sclerotial development		16
Ustiloxin B	31	RiPPS	Yes	15	?		10,17

[a]Some clusters produce more than one metabolite.
[b]Based on Georgianna et al.[8] numbering system. NA-not applicable as the kojic acid cluster was not identified by SMURF analysis.
[c]As defined experimentally or as defined in Georgianna et al.[8].

widely-used technique, especially for preliminary metabolite investigation[123]. Normal-phase TLC is commonly used for mycotoxin analysis and utilizes non-polar solvent systems to separate metabolites over silica gel adsorbent. TLC plates pretreated with a fluorescent indicator allow UV absorbing compounds to be easily detected. Non-UV active and non-fluorescent compounds can be detected by treating the plate with various developing reagents[124]. High-performance liquid chromatography (HPLC) affords better separation, usually on a C18 reverse-phase column, and when paired to a diode-array detector (DAD) provides diagnostic UV spectra for each peak[125,126]. Fungi can be identified using chemotaxinomic data acquired from organic fungal extracts run on an HPLC-DAD instrument. Coupling HPLC-DAD to a mass spectrometer (MS) provides more information for identification[127,128]. High resolution mass spectrometry (HRMS) allows molecular formula estimation and simplifies dereplication[129]. Databases of characterized natural products searchable by monoisotopic mass, such as Antibase, can assist in dereplication[130]. HPLC-DAD-MS dereplication of

doi: 10.14252/foodsafetyfscj.2017024

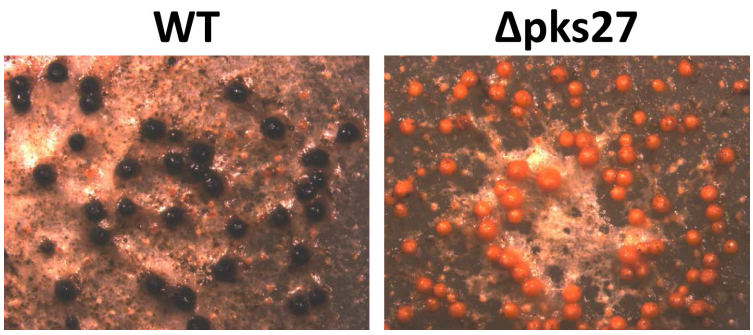


Fig. 1. Microscopic examination of sclerotia. Sclerotia production in *A. flavus* SRRC1582 wild-type (WT) and a Δ*pks27* mutant was observed using an Olympus SZH10 stereomicroscope (x96 magnification) and Nikon DS-Qi1 camera. Strains were grown on GMMS agar for 10 days in the dark at 30°C. Note loss of dark pigmentation in sclerotia of the Δ*pks27* mutant due to abrogation of production of asparasone A.

SM production produced by genome-sequenced strains of *A. oryzae* (RIB40) and *A. flavus* (NRRL 3357) revealed that, although bioinformatics analysis shows 99.5% gene homology between the strains, the overall chemical profiles differed significantly[131]. Orbitrap HRMS and multiple stage mass spectrometry (MS^n) was used in conjunction with SIEVE software to identify differentially expressed metabolites in control and mutant *A. flavus* strains (see section 5–1)[132]. Similar analytical methodology was developed using ultra-high-performance liquid chromatography (UHPLC)-Orbitrap HRMS that allows untargeted determination of fungal metabolites and led to the characterization of *A. flavus* cluster 23 metabolites (see section 5–2)[133].

One-dimensional (1D)-nuclear magnetic resonance (NMR) data, usually ^{1}H and ^{13}C spectra, are often used to confirm metabolite identification. Structure elucidation, particularly atom connectivity, of new natural products is reliant on various 2D-NMR techniques[134]. Purification of metabolites is usually required for structure elucidation, however, recently, several metabolites have been identified and structurally characterized using NMR without having been isolated in pure form[135]. 2D-NMR analysis of unpurified extracts can also be used to investigate an organisms' metabolome[136]. Differential analysis by 2D-NMR spectroscopy (DANS)[137,138] was employed to examine the gliotoxin biosynthesis pathway (*gli*) in *A. fumigatus*, which revealed many new *gli*-dependent metabolites[139].

5. Experimentally Identified *A. flavus* Secondary Metabolite Gene Clusters

5-1. Asparasone Cluster

Asparasone A, a PKS-derived 1,3,6,8-tetrahydroxy-2-(1′-hydroxy-3′-oxobutyl)-anthraquinone was shown to be a precursor molecule required for the formation of the dark pigment associated with the outer rind of sclerotia[52]. Asparasone A was first described in *A. parasiticus* extracts as a pigment that was structurally related to versicolorin intermediates involved in the biosynthesis of AFs and demonstrated to possess antiinsectan properties[108]. The asparasone A gene cluster consists of four genes, a polyketide synthase (AFLA_082150, *pks27*), a Zn(2)-Cys(6)-type transcription factor (AFLA_082140, *znf27*) and a putative high-affinity glucose transporter (AFLA_082160, *mfs1*) and MFS transporter (AFLA_082170, *mfs2*)[52]. *A. flavus* Δ*pks27* mutants produced greyish-yellow sclerotia rather than having the dark brown pigment presented by control strains (**Fig. 1**). Complementation of the Δ*pks27* mutant with a wild-type copy of *pks27* restored production of dark-pigmented sclerotia. Production of sclerotia was significantly reduced in the Δ*pks27* mutant compared to that of the wild-type. Ultra-high performance liquid chromatography and mass spectrometry revealed that asparasone A (m/z (M-H)=357.0612) was still being produced by the Δ*znf27* mutant though at significantly reduced levels which probably explained the reduction in observed sclerotial pigmentation. While function of the putative *mfs1 and mfs2* transporters was not confirmed, they were considered to be a part of the asparasone cluster due to their coordinate regulation with *pks27* by *znf27*.

The characteristic dark pigmentation associated with most fungal sclerotia was previously thought to be due to a process

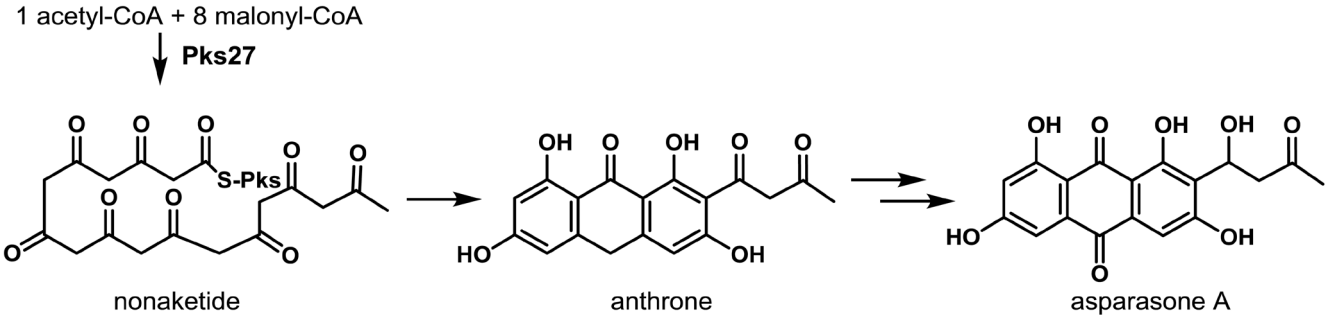


Fig. 2. Proposed biosynthesis of asparasone A in *A. flavus*. Pks27 assembles the linear nonaketide from an acyl started unit and eight malonyl units. Cyclization and release from the PKS affords the anthrone. The anthrone is oxidized to the anthraquinone and one ketone moiety reduced by unknown enzymes that are predicted to be similar to those in the aflatoxin biosynthesis gene cluster. Adapted from Cary et al[52].

of melanization. However, the study of Cary et al.[52] showed the dark brown pigments present in *A. flavus* sclerotia are the product of PKS27-derived anthraquinones rather than typical tetrahydronapthalene-based melanins (**Fig. 2**). The presence of the asparasone A-based anthraquinone pigment in *A. flavus* sclerotia was shown to provide resistance to both biotic and abiotic stressors. In both choice and no choice feeding assays with the sap beetle *Carpophilus freemani*, a preference for consumption of sclerotia produced by the Δ*pks27* mutant compared to control sclerotia was observed indicating that the dark pigment functions as an insect antifeedant. Exposure of sclerotia to UV irradiation or high temperatures resulted in decreased viability of sclerotia from the Δ*pks27* mutant compared to the control strain.

5-2. Leporin Cluster

Of the 55 *A. flavus* SM gene clusters identified by SMURF analysis, two were found to harbor hybrid PKS-NRPS backbone genes. One of these clusters was shown to be responsible for the production of the indole tetramic acid, cyclopiazonic acid[140] while the second cluster produced a family of 2-pyridones known as leporins[15]. TePaske et al.[141] reported on the production of the antiinsectan compound, leporin A, in the fungus *Aspergillus leporis*, however there had been no previous reports of leporin production in *A. flavus*. In addition to the PKS-NRPS (AFLA_066840, *lepA*), the leporin cluster was predicted to encode an additional 14 genes that could be involved in metabolite production[15]. An *A. flavus* Δ*lepA* mutant showed no significant differences in metabolite production compared to that of an isogenic control strain, indicating that the genes responsible for leporin biosynthesis were not being expressed or expressed at extremely low levels. However, overexpression of a putative Zn(2)-Cys(6) cluster-specific transcriptional regulatory gene, *lepE* (AFLA_066900) resulted in transformants producing a distinctive orange-red pigment (**Fig. 3**). Liquid chromatography and high resolution mass spectrometry (LC-HRMS) analysis of the OE::*lepE* extracts revealed a metabolite of *m/z* (M+H)=352.1902 that represented a demethylated leporin A precursor designated leporin B[15]. Additionally, a metabolite of *m/z* (M+H)=336.1959 was also produced that represented the N-demethoxy derivative of leporin A, designated leporin C. While leporin B had been previously reported to be produced by an unidentified fungus[142], this was the first report of production of leporin C. Interestingly, metabolites were identified in the OE::*lepE* strain that were consistent with homodimers of both leporin B and C.

The biosynthesis of leporin B and C was elucidated using LC-HRMS analysis of extracts from knockout mutants of the leporin cluster genes in the *A. flavus* OE::*lepE* background[15] (**Fig. 4**). The products of both *lepA* (PKS-NRPS) and *lepG* (enoyl reductase) are responsible for fusion of phenylalanine with a hexaketide and subsequent release of the stable tetramic acid precursor, pre-leporin C. The putative cytochrome P450, LepH, an enzyme demonstrating significant homology to the ring expansion cytochrome P450s found in the 2-pyridones, tenellin and bassianin, was shown to be required for the ring expansion step. LepF, a putative short chain dehydrogenase/reductase, was hypothesized to reduce the acyl carbonyl to an alcohol[143]. Interestingly, although *lepI* was predicted to encode a putative methyltransferase, elegant *in vitro* studies by Tang and coworkers[143] showed that LepI catalyzed an S-adenosyl-L-methionine (SAM)-dependent inverse electron demand hetero-Diels–Alder (HDA) reaction forming leporin C. In addition to the HDA reaction, LepI was shown to possess retro-Claisen activity, which allows the enzyme to convert a byproduct of the Diels–Alder reactions to leporin C. Leporin C is then further oxidized by the N-hydroxylase, LepD, to form leporin B. The function of LepA, G, H, F, I and D were confirmed by heterologous reconstitution in *A. nidulans*; the function of LepF and LepI were investigated *in vitro* using recombinant protein expressed from *S. cerevisiae*

doi: 10.14252/foodsafetyfscj.2017024

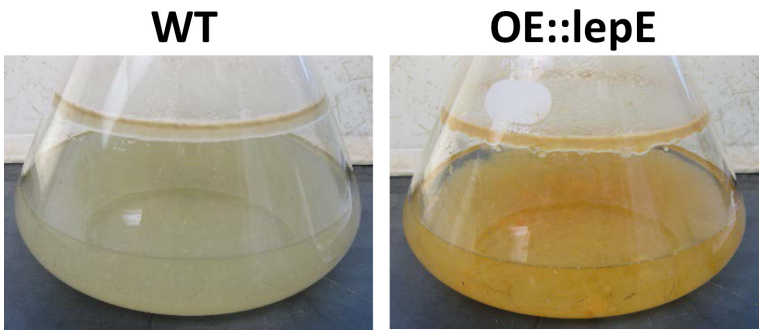


Fig. 3. Production of leporins by *A. flavus*. Images of broth cultures of an *A. flavus* CA14 OE::*lepE* strain and CA14 wild-type (WT) following 2 days growth on Czapek-Dox broth with agitation in the dark at 30°C. Note orange-red pigmentation of the OE::*lepE* culture broth indicative of the formation of leporin B-iron complexes.

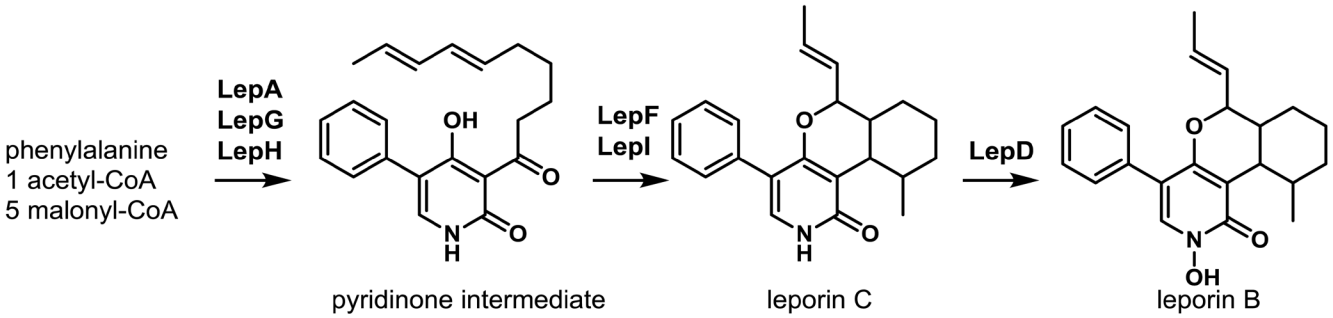


Fig. 4. Proposed biosynthesis of leporin B. The hybrid PKS-NRPS LepA and the LepG enoyl reductase form a tetramic acid intermediate (not shown), which is converted to a 4-hydroxypyridinone intermediate by the ring-expanding P450 LepH. LepF reduces the ketone in the intermediate to an alcohol. The S-adenosyl-L-methionine (SAM)-dependent enzyme LepI catalyzes a stereoselective dehydration of the alcohol followed by an inverse electron demand hetero-Diels–Alder reaction forming leporin C. The LepD P450 forms the hydroxamic acid moiety through N-hydroxylation resulting in leporin B. Adapted from Cary et al.[15] and Ohashi et al.[143].

and *E. coli*, respectively[143].

While 2-pyridones are normally colorless, it is believed that leporin B, in the presence of an atom of Fe(III) is oxidized to form a highly stable leporin B trimer with an atom of iron (0) (*m/z* = 1107, iron(0)-trioxyleporin B) which results in the observed orange-red colony pigmentation. It was not determined if oxidation of leporin B to form the trimer is self-catalyzed or catalyzed by another enzyme residing within or outside of the leporin gene cluster. Such a trimeric iron chelate had not been previously described for a 2-pyridone and unlike typical iron-chelating siderophores, the leporin B-iron complex was highly soluble in organic solvents thus representing the first siderophore-like molecule with this property to be produced by a PKS-NRPS.

Though leporin A from *A. leporis* was shown to have antiinsectan properties there were no previous reports on the insecticidal properties of leporins B or C. In no-choice insect feeding studies performed with a preparation of the leporin B-iron chelate (LIC) compound extracted from the OE::*lepE* strain, a significant reduction in growth of first instar larvae of Fall armyworms (*Spodoptera frugiperda*) and Corn earworms (*Helicoverpa zea*) was observed[15]. In choice assays, both caterpillar species were observed feeding more frequently on the control disks than 0.1% LIC disks, and consumed significantly less of the LIC diet disks than the control diet disks. These results indicated that the LIC was acting as an antifeedant with little evidence of antiinsectan properties. Alterations in the developmental programs were also noted between the OE::*lepE* strain compared to the control[15]. A 10-fold reduction in conidia production was observed in the OE::*lepE* strain and sclerotial production was either reduced or delayed depending on the growth medium used compared to the control. Studies have shown that conidiation and sclerotial differentiation in filamentous fungi including *A. flavus* are induced by oxidative stress[144,145]. Cary et al.[15] speculated that the large quantities of leporin B produced by the OE::*lepE* strain may have led to an increase in sequestration of iron, in the form of the leporin B-iron chelate, thus reduc-

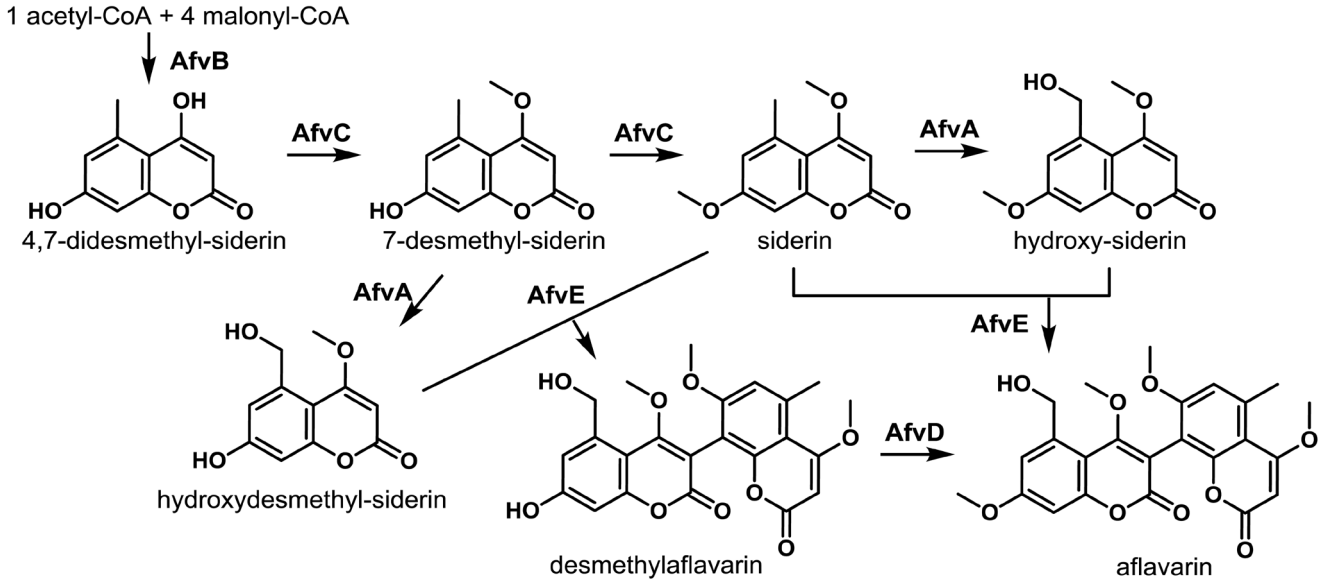


Fig. 5. Proposed biosynthesis of aflavarin. The PKS AfvB assembles 4,7-didesmethyl-siderin from an acyl started unit and four malonyl units. AfvC can perform subsequent methylations affording siderin. Hydroxylation by AfvA gives hydroxy-siderin, which can be oxidatively coupled with siderin by the AfvE P450 to form aflavarin. An alternate pathway to aflavarin is possible by an AfvE-catalyzed coupling of hydroxydesmethyl-siderin and siderin followed by a methylation of desmethylaflavarin by AfvD. Adapted from Cary et al.[29].

ing oxidative stress caused by excess free iron. The reduction in oxidative stress levels, in turn, resulted in a decrease in the production of both conidia and sclerotia.

5-3. Aflavarin Cluster

Comparative transcriptomics of an *A. flavus* Δ*veA* mutant and isogenic control strain led to the identification of the aflavarin gene cluster in which all but one of the genes were significantly down-regulated in the mutant in at least one of the conditions assayed[29]. Like many previously characterized *A. flavus* SMs, aflavarin was isolated from sclerotia and shown to exhibit antiinsectan activity however the biosynthetic genes responsible for its production were unknown[146]. SMURF analysis predicted the aflavarin cluster to be composed of a backbone PKS gene (AFLA_108550, *afvB*) encoding an N terminal beta-ketoacyl synthase (KS) domain with an adjacent acyl transferase (AT) domain and a C-terminal malonyl CoA-acyl carrier protein transacylase (ACP) domain. Other *veA*-dependent genes present in this cluster included an O-methyl transferase (AFLA_108560, *afvC*), methyl transferase (AFLA_108570, *afvD*), cytochrome P450 (AFLA_108580, *afvE*), and a NADH oxidase (AFLA_108540, *afvA*). A blastp search of the NCBI and the Broad Aspergillus Comparative Genome databases indicated that the genes constituting the aflavarin gene cluster in *A. flavus* were highly conserved in *A. oryzae*, *A. niger* and partially conserved in *A. clavatus* and *A. terreus.*

Comparative analysis of the metabolomes of an *A. flavus* control and Δ*afvB* PKS mutant using high resolution mass spectrometry allowed identification of a series of bicoumarins that were present in the control and absent in the mutant[29]. The major differentially produced metabolite had a calculated mass of 455 which was identical to the mass of aflavarin and this was subsequently confirmed by LC/MS comparison to an authentic sample of aflavarin. Knockout of the cluster biosynthetic genes allowed determination of their roles in aflavarin synthesis (**Fig. 5**). Deletion of the O-methyltransferase (*afvC*) gene, involved in the O-methylation of the PKS-derived pentaketide, didesmethyl-siderin, totally abolished the biosynthesis of the monomeric siderin precursors that are subsequently dimerized to form the final bicoumarin products. Disruption of the cytochrome P450 monooxygenase (*afvE*) led to accumulation of the monomeric coumarins and loss of bicoumarin production, proving that the cytochrome P450 monooxygenase is responsible for phenolic coupling reactions. Inactivation of the *afvD* methyltransferase was found to be responsible for the final O-methylation steps following formation of the bicoumarins. The putative NADH oxidase (*afvA*) was proposed to be involved in hydroxylation of siderin and 7-O-demethyl siderin to their respective hydroxylated analogues.

In addition to its previously demonstrated antiinsectan properties, it was also shown that production of aflavarin is necessary for normal levels of sclerotial production. While no cluster-specific transcriptional activator gene was present in aflavarin cluster, there were a number of putative binding sites for stress-responsive DNA binding proteins as well the

doi: 10.14252/foodsafetyfscj.2017024

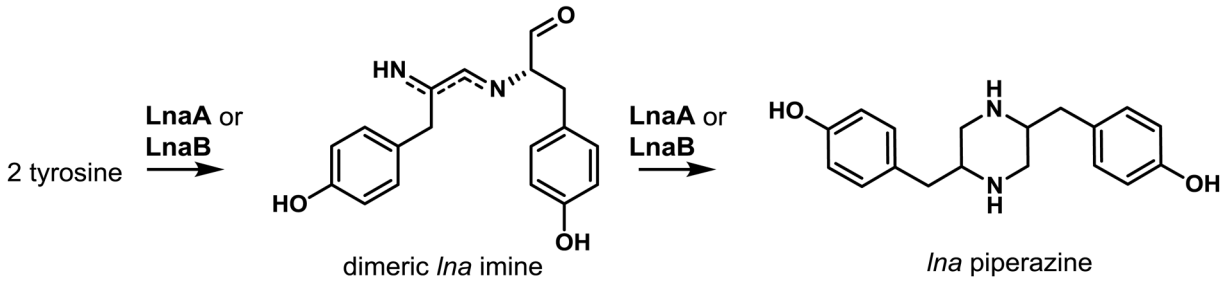


Fig. 6. Proposed biosynthesis of piperazines. The NRPS-like enzymes LnaA and LnaB catalyze the reduction of tyrosine and form an imine dimer. LnaA and LnaB can then reduce the imine dimer to a piperazine. LnaB and LnbB can form a morpholine analog from the imine dimer intermediate, while LnaC and LnbC are proposed to oxidize the imine dimer to a pyrazine analog (not shown). Adapted from Forseth et al.[16].

AF cluster-specific transcriptional activator, AflR. This suggested that there may be regulatory cross-talk between these 2 clusters. (**Fig. 6**)

5-4. Piperazine Clusters

Two clusters were identified in *A. flavus* named lna (chromosome 6) and lnb (chromosome 8) that were shown to be primarily involved in the biosynthesis of two piperazines as well as a number of other derivatives[16]. The genetic makeup of the two clusters is quite similar being composed of genes encoding a putative NRPS protein (*lnaA*/*lnbA*), and a number of genes likely to encode tailoring enzymes such as an NMR-like protein (*lnaB*/*lnbB*), cytochrome P450s (*lnaC*/*lnbC*), alcohol dehydrogenase/NADH:flavin oxidoreductase (*lnaE*/*lnbE*), and a transporter (*lnaF*/*lnbF*). The lna cluster also contains a second putative P450 enzyme gene (*lnaD*) while the lnb cluster contains a gene encoding a hypothetical protein not present in the lna cluster. The LnaA and LnbA NRPSs share a fairly high level of amino acid identity (58%) and canonical adenylation, acyl carrier protein and thioester reductase domains but lack a condensation domain. No cluster-specific transcriptional regulator genes were found in either the lna or lnb clusters. However, both clusters were shown to be under the control of LaeA, a global regulator of fungal development, secondary metabolism and virulence[86,147]. Searches of fungal genome databases indicated that LnaA-like proteins are present a number of *Aspergillus* species (though not specified) and in the genomes of the basidiomycetes, *Serpula lacrymans* and *Heterobasidion annosum*[16].

Comparative metabolomics of *A. flavus* wild-type, deletion, and *lnaA* overexpression (OE) strains allowed identification of two major piperazine metabolites and eight other lna cluster-derived metabolites that had not been previously reported in aspergilli or other fungi[16]. Both LnaA and LnbA proteins were shown to activate tyrosine but because they lacked condensation domains, there was no peptide bond formation as confirmed by their inability to be activate an L-Tyr-L-Tyr dipeptide. Instead it was postulated that the C-terminal reductase domains of LnaA and LnbA act to reduce L-Tyr to its aldehyde. This suggested that the lna cluster NRPSs function as amino acid reductases characteristic of Lys2-type reductases involved in fungal L-lysine biosynthesis[148]. The resulting L-Tyr aldehyde was proposed to dimerize and be subsequently further reduced to piperazines and morpholine compounds by the activities of the NMR-like reductase LnaB/LnbB. While the function of the LnaC, LnaD or LnbC oxidases was not determined it was speculated that one or all may be involved in the conversion of the Tyr dimer to a pyrazine. Of particular interest was the finding that LnaB can participate in the reduction of LnaA-derived intermediates indicating that the lna and lnb clusters encode partially redundant biosynthetic pathways capable of sharing biosynthetic intermediates.

Suppression of *lnaA* and *lnbA* expression in *A. flavus* resulted in significant reduction in sclerotial formation compared to the wild-type suggesting a role for the SM products of these clusters in fungal development[16].

5-5. Aflatrem Clusters

Similar to piperazine biosynthesis, the genes required for the synthesis of the potent tremorgenic toxin, aflatrem, are also located at loci present on two different chromosomes (5 and 8) in *A. flavus*[140]. Aflatrem and its isomer, β-aflatrem, are examples of indole-diterpenes (IDTs), a class of structurally-diverse SMs that are found mainly in *Aspergillus* and *Penicillium spp.* as well as *Epichloë*. Identification of the genes required for aflatrem biosynthesis (*atm*) in *A. flavus* and *A. oryzae* was made possible through comparative genomics with genes from *Penicillium paxilli* required for the production of the indole-ditepene, paxilline[109,149]. As observed in *A. flavus*, the genes required for paxilline biosynthesis (*pax*) are clustered in 2 loci present on separate chromosomes. A total of three *pax* gene homologs (*atmG*,

atmM and *atmC*) comprise the *A. flavus* ATM1 locus while *atmD*, *atmQ*, *atmB*, *atmA* and *atmP* comprise the ATM2 gene cluster locus. The presence of these *pax* gene homologs in *A. flavus* provided support for the biosynthesis of aflatrem proceeding via a paxilline intermediate.

Indole-diterpenes are all derived from a common core structure consisting of a tetracyclic diterpene derived from the activities of a geranylgeranyl diphosphate synthase (GGPPS, PaxG/AtmG) and geranylgeranyl transferase (GGT, PaxC/AtmC) that combines GGPP with an indole moiety derived from indole-3-glycerol-phosphate to form 3-geranylgeranyl indole (GGI)[140]. GGI is then converted to an epoxide by the action of an epoxidase (PaxM/AtmM) and subsequently transformed by an IDT cyclase (PaxB/AtmB) to the first stable intermediate, paspaline[150,151]. In *A. flavus*, paspaline is then converted to paspalinine by the action of the cytochrome P450 monooxygenases AtmP and AtmQ followed by subsequent conversion to aflatrem by the prenyltransferase AtmD. Interestingly, the chemical diversity that is characteristic of IDTs is due in large part to the IDT cyclases (IDTCs). Using a yeast host, Tang et al.[151] were able to propose mechanisms by which *A. flavus* utilizes unique IDTCs to produce both aflatrems and the IDT, aflavinine (**Fig. 7**). They proposed that AtmG/C/M produces a common epoxide intermediate which is then converted to paspaline by the aflatrem cluster IDTC, AtmB, thus leading to subsequent formation of aflatrem. Based on the ability of *A. flavus* to also produce aflavinine, Tang et al.[151] performed a phylogenetic analysis of all *atmB* homologs in *A. flavus* to identify candidate IDTCs that may be responsible for formation of aflavinine. When they introduced the standalone (not associated with a typical SM gene cluster) *atmB* homolog, *afB*, into a yeast host expressing *atmG*/*C*/*M*, the major product was shown to be aflavinine. These findings indicated that the standalone IDTC, AfB, catalyzes an alternate set of cyclization reactions leading to the production of aflavinine. How the fungus regulates expression of IDTCs such as *atmB* and *afB* to selectively control the ratios of aflatrems to aflavinines produced is unclear but it may have to do with the desired biological activity inherent in the particular IDT. Interestingly, the yeast studies indicate that the product of the *atmA* gene is not required for aflatrem or aflavinine biosynthesis.

Expression of *atm* genes at both ATM loci were shown to be coordinately controlled despite their presence on separate chromosomes. However, no cluster-specific transcriptional regulatory genes were found in or near either of the ATM loci and this was also the case for the *pax* loci in *P. paxilli*[140]. This indicated that control of expression of *atm* genes probably occurs at the global level. In fact, it was shown that the global regulators of *A. flavus* development and secondary metabolism, VeA and LaeA, are both required for production of wild-type levels of aflatrem[86,90].

Aflatrems and aflavinines are both associated with sclerotia in *A. flavus*[152]. Aflatrem was shown to be a potent mammalian tremorgen[153] while aflavinines demonstrated antiinsectan properties[152]. The biological advantage that aflatrem imparts on the fungus is not clear though β-aflatrem has been shown to have some antiinsectan activity[146]. The mechanism of action of aflatrems and aflavinines remains to be determined but their antifeedant/antiinsectan properties suggest a role to increase survival of sclerotial propagules in the field from insect predation, thus allowing eventual germination to produce more fungal spores for dissemination.

5-6. Ustiloxin B Cluster

The proliferation of next generation sequencing of fungal genomes coupled with web-based bioinformatics tools (e.g. SMURF and antiSMASH) has been a major driver in the identification of secondary metabolic gene clusters in filamentous fungi. However, these prediction algorithms are based in large part on the presence of typical SM cluster backbone genes such as PKSs and NRPSs. A novel algorithm created by Umemura et al.[10], designated MIDDAS-M was able to predict fungal SM gene clusters lacking a backbone gene based on integration of annotated genome and transcriptomic data. Using this approach, they were able to identify a gene cluster in *A. flavus* predicted to consist of 18 genes, none of which were identified as a typical cluster backbone gene. Comparative metabolomics of gene knockout mutants of this cluster compared to a control strain identified a metabolite of m/z (M+H)=646.240 that corresponded to the antimitotic cyclic peptide, ustiloxin B.

Ustiloxins were originally identified in the rice pathogen, *Ustilaginoidea virens*, but were not known to be produced by *A. flavus*[154,155]. These compounds are tetrapeptides with ustiloxin B consisting of the amino acids Tyr-Ala-Ile-Gly (YAIG). Enzymes encoded by the gene cluster cyclize the tetrapeptide at the side chains of Tyr and Ile and catalyze side chain modifications and linking of a non-proteinogenic amino acid, norvaline, to the Tyr moiety[17] (**Fig. 8**). Ustiloxin B was initially thought to be the product of a NRPS-like enzyme, but no NRPS-like genes were present in or near the gene cluster. Upon further examination, the cluster gene *ustA*, was predicted to encode a protein containing an N-terminal signal sequence and 16-fold repeated peptide region containing YAIG repeats which represent the exact tetrapeptide sequence of ustiloxin B. Biosynthesis was proposed to proceed via processing of the UstA precursor protein by a Kex2 protease not present in the cluster that cleaves at nearby Lys-Arg residues on the C-terminal side of the YAIG tetrapeptide[17].

doi: 10.14252/foodsafetyfscj.2017024

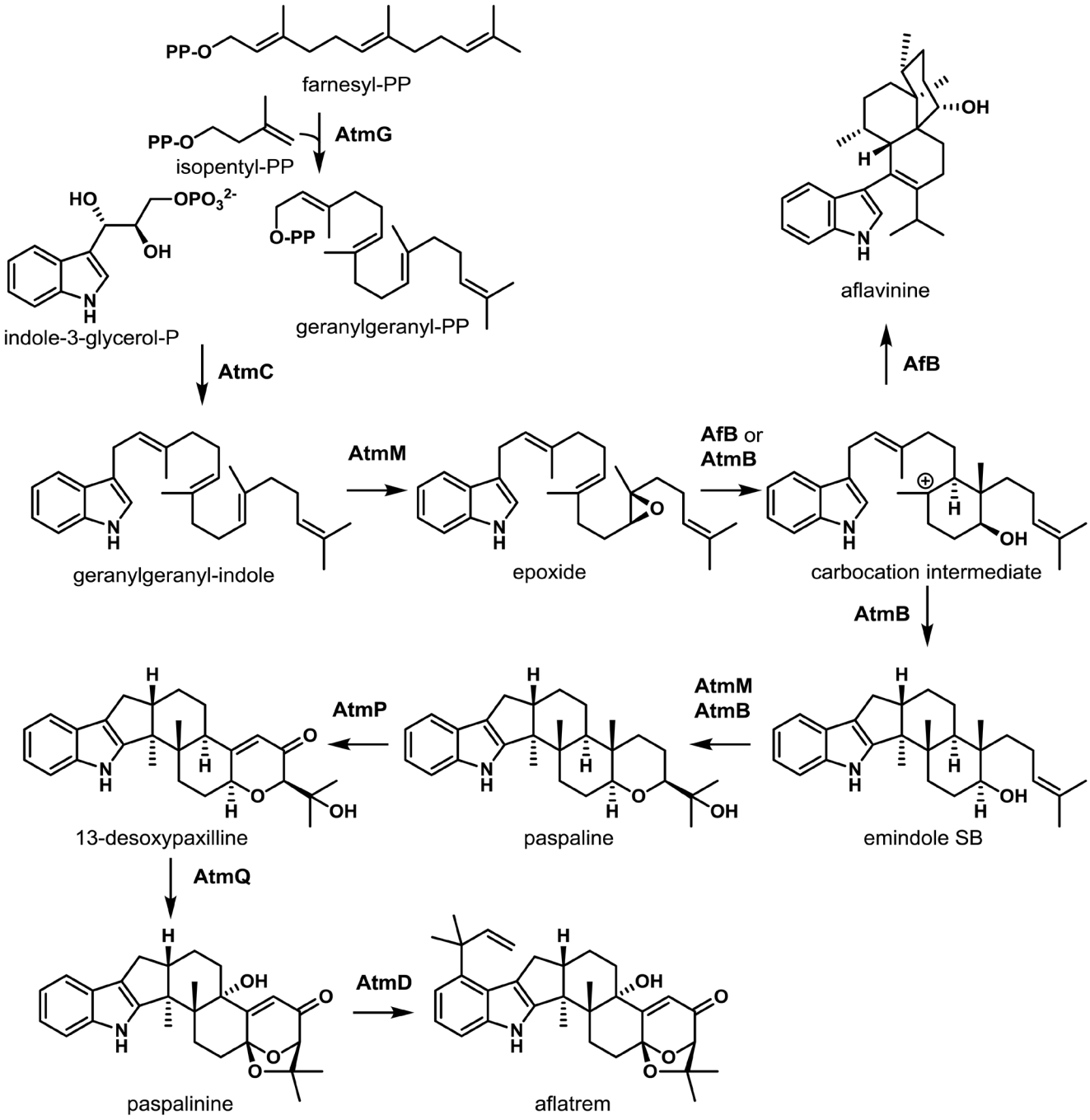


Fig. 7. Proposed biosynthesis of indole diterpenes in *A. flavus*. Two indole diterpene cyclases (AtmB and AfB) work in conjunction with the epoxidase AtmM to produce diverse chemical structures from a common intermediate. Genes encoding for biosynthesis of the epoxide intermediate (AtmG, AtmC, and AtmM) are clustered together (*ATM1*). Genes encoding for biosynthesis of paspaline, paspalinine, and aflatrem (AtmB, AtmP, AtmQ and AtmD) are also clustered together (*ATM2*), while the gene encoding AfB, responsible for aflavinine biosynthesis, is not clustered with either *ATM1* or *ATM2*. Adapted from Nicholson et al.[140] and Tang et al.[151].

Subsequent work in *A. oryzae* confirmed that inactivation of the *kexB* endopeptidase gene resulted in almost complete loss of ustiloxin B[156]. The partially processed pre-protein is then believed to be processed in a stepwise manner by the products of *ustP* and *ustH*, both encoding peptidases, resulting in generation of the mature YAIG tetrapeptide. Prior to removal of the N-terminal leader peptide by UstP/UstH, the pre-protein is believed to be modified and cyclized by the action of the monooxygenases UstF1/UstF2 and UstQ (tyrosinase), respectively, to yield the intermediate ustiloxin F. The intermediate is then further modified by methylation (UstM), linkage of the norvaline to the Tyr residue (UstF and UstS) and oxygenation of the sulfur group (UstC). The functions of a number of genes considered as part of the

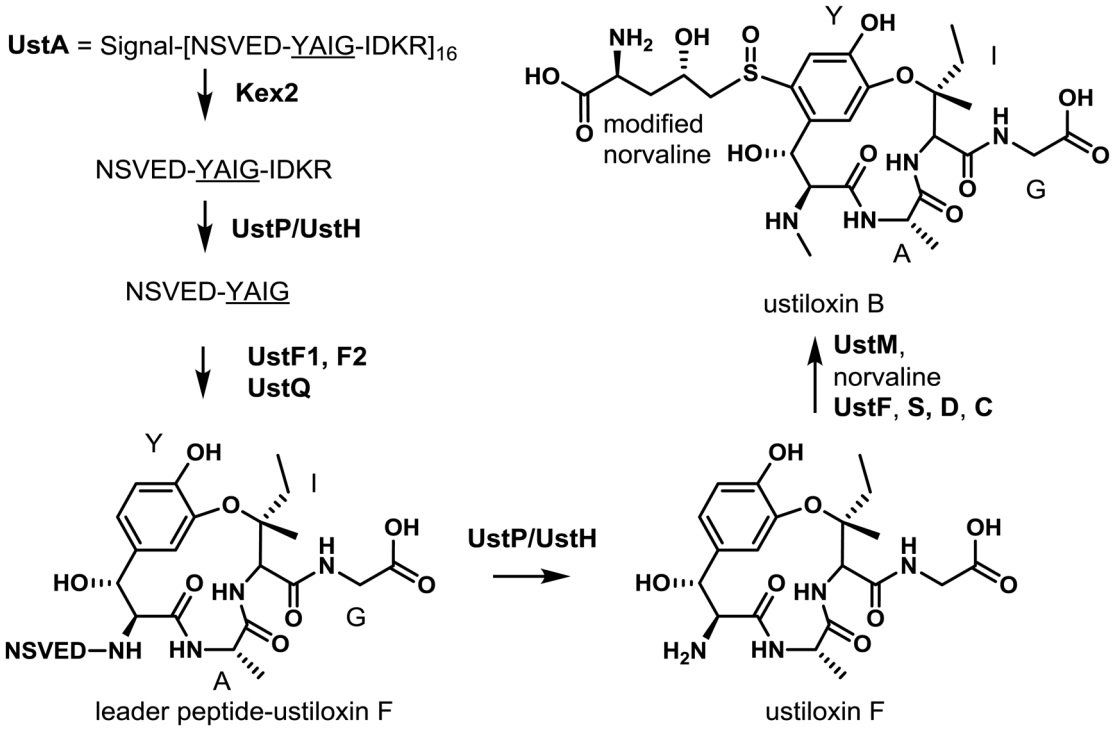


Fig. 8. Proposed biosynthesis of ustiloxin B. UstA is processed by the endoproteinase Kex2 at the C-terminus in the Golgi apparatus resulting in 16 copies of the precursor peptide. The C-terminus of each precursor peptide is then cleaved by peptidases UstP and UstH to afford a nonapeptide containing a leader and core peptide (underlined). While still attached to the leader peptide, the core YAIG peptide is cyclized. The leader peptide is then cleaved by UstP and UstH affording ustiloxin F. Methylation by UstM is follow by attachment and modification of norvaline by UstF, S, D, and C yielding ustiloxin F. Adapted from Umemura et al.[17].

ustiloxin B gene cluster were not determined in the study of Umemura et al.[17]. The absence of an NRPS-like gene in the cluster along with a predicted protein (UstA) harboring 16 short YAIG repeat peptide regions and an N-terminal signal peptide suggested that ustiloxin B is biosynthesized through a ribosomal peptide synthetic pathway (RiPS) not previously described in *A. flavus* or other Ascomycetes[17].

A subsequent study by Nagano et al.[14] showed that deletion mutants of *ustQ* or *ustYa*/*ustYb* failed to produce the pathway intermediate ustiloxin F, suggesting that all three gene products are involved in cyclization of the intermediate. Interestingly, Nagano et al.[14] screened *Aspergillus* genome sequences for the presence of the *ustYa*/*ustYb* and *ustA* gene homologs and were able to predict the presence of 94 ribosomal peptide precursor candidate genes that could be classified into 40 types having a variety of core peptide sequences. From this computational analysis they were able to experimentally identify a novel bicyclic peptide designated asperipin-2a in *A. flavus*.

Despite their ability to inhibit polymerization of purified porcine microtubule proteins as well as mitosis in human cell lines but not bacteria or fungi, the role of ustiloxin B in the biology of *A. flavus* remains to be determined[154].

5-7. Ditryptophenaline Cluster

The ditryptophenaline (DTP) cluster is responsible for the production of the dimeric diketopiperazine alkaloid, (−)-ditryptophenaline, that is derived from two molecules of tryptophan and phenylalanine[157]. Comparative metabolomics of putative *A. flavus* DTP cluster gene (*dtp*) mutants with a control strain enabled determination of the function of the cluster genes. The cluster was found to be composed of three genes encoding a NRPS (*dtpA*) that condenses L-tryptophan and L-phenylalanine together to form the diketopiperazines core; an N-methyltransferase (*dtpB*) that methylates the core structure; and a cytochrome P450 (*dtpC*) that is likely responsible for cyclization and dimerization of the methylated diketopiperazine to form DTP (**Fig. 9**). Though DTP has been shown to have weak activity as an inhibitor of the neurotransmitter, substance P, in animal models, its function in the biology of *A. flavus* is not known[110].

5-8. Kojic Acid (KA) Cluster

Though not originally confirmed in *A. flavus*, the KA gene cluster was experimentally identified in its close relative, *A. oryzae*[111]. Use of comparative transcriptomics of *A. oryzae* cultures grown under KA-inducing and non-conducing

doi: 10.14252/foodsafetyfscj.2017024

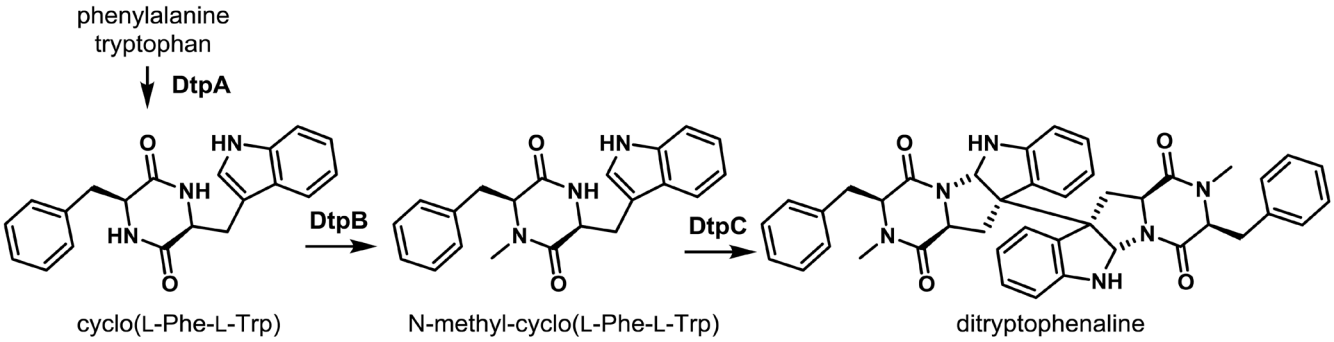


Fig. 9. Proposed biosynthesis of dityrptophenaline. The NRPS DtpA forms the diketopiperazine cyclo(L-Phe-L-Trp) from phenylalanine and tryptophan. DtpB methylates cyclo(L-Phe-L-Trp) and the P450 DtpC catalyzes dimerization affording ditryptophenaline. Adapted from Saruwatari et al.[157].

conditions, allowed the identification of a limited number of genes that may be involved in KA biosynthesis. Deletion of these candidate genes in *A. oryzae* followed by growth on media that is diagnostic for production of KA allowed identification of two deletion mutants that could no longer produce KA. The genes knocked out in these two mutants mapped within 3 kb of one another on the *A. oryzae* genome suggesting that a small gene cluster was responsible for KA biosynthesis. One of the genes (AO090113000136; *A. flavus* AFLA_096040) designated *kojA*, encodes a predicted FAD-dependent oxidoreductase while the other, *kojT* (AO090113000138; *A. flavus* AFLA_096060) is a major facilitator superfamily transporter. KojA is believed to function in the oxidation of glucose to KA though other as of yet unidentified genes may be required for complete conversion of glucose to KA[111] (**Fig. 10**). Located between *kojA* and *kojT* is a gene designated *kojR* (AO090113000137; *A. flavus* AFLA_096050) that encodes a Zn(2)-Cys(6) transcription factor.

The earlier studies of Terabayashi et al.[111] had shown when sodium nitrate was added to KA production medium, KA biosynthesis was inhibited in *A. oryzae*. The negative impact of sodium nitrate on KA production was supported by deletion of *nrtA*, encoding a transporter essential for sodium nitrate uptake in *A. oryzae*[158]. Deletion strains of *nrtA* were still be able to produce KA even in the presence of sodium nitrate and the expression of *kojA* and *kojT* were significantly higher in the *nrtA* mutant than the control in the presence of sodium nitrate.

Though the contribution of KA to the biology of the producing fungus is not clear at this time it appears that it may be involved as an insect antifeedant or perhaps as an antioxidant that reduces the ability of insect oxidative enzymes to detoxify AFB_1 or host plant-based antiinsectan compounds such as nicotine[159]. Compared to AFB_1, kojic acid was shown to be relatively non-toxic to mice but in

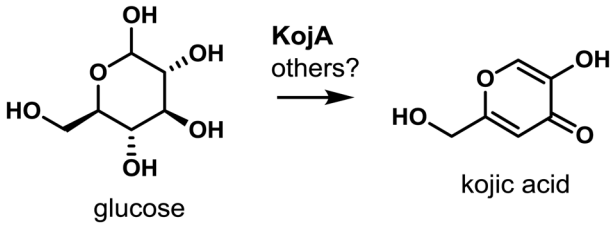


Fig. 10. Proposed biosynthesis of kojic acid. The putative FAD-dependent oxidoreductase KojA is predicted to form kojic acid from glucose, possibly in conjunction with other enzymes not encoded in the gene cluster. Adapted from Terabayashi et al.[111].

insect feeding studies in combination with AFB_1, it appeared to act synergistically causing increased mortality compared to feeding of either metabolite alone[159]. The ability of KA to chelate iron and other cations may indicate that it is involved in homeostasis of these metals.

6. Conclusions

The advent of fungal genomics, bioinformatics and advanced analytical chemical techniques has resulted in a rapid expansion of the numbers of identified *A. flavus* SM gene clusters and their affiliated metabolites. While it took the better part of two decades to identify the aflatoxin gene cluster and the enzymes required for production of aflatoxins, genome sequencing and annotation coupled with modern molecular biological, bioinformatic and analytical techniques have allowed an additional nine *A. flavus* metabolites and their associated gene clusters and enzymes/regulatory genes to be described, all within a period of less than a decade. Though metabolites have been linked to most of the more readily tractable SM gene clusters in *A. flavus*, there remains a number of silent gene clusters for which characterization of their SM product's form and function is lacking.

There is a growing body of evidence that points to signifi-

cant roles of SMs in *A. flavus* biology and therefore it is critical that approaches are developed to activate silent SM gene clusters so that their impact on the physiology of the fungus can also be ascertained. While a number of techniques have been used successfully to activate silent gene clusters in the model fungus, *A. nidulans*, as well as other *Aspergillus* spp., experiments of this nature are lacking for the most part in *A. flavus*. More information must be gleaned on regulatory mechanisms of SM production in *A. flavus* such as global and cluster-specific regulators, signaling pathways, epigenetic regulation, and how these respond to the fungus' environment. Evidence for regulatory cross-talk between *A. flavus* gene clusters and their ability to share biosynthetic intermediates has opened up new avenues of investigation into the regulation of SM gene cluster biosynthetic machinery. It is imperative that laboratory observations on the biological function of SMs also be translated to the field with respect to the interaction of *A. flavus* with the environment, especially during the host plant-fungus interaction. Additionally, little is known about the role of SMs on the interaction of *A. flavus* with other microbes that constitute the microbiome of the host plant and surrounding soil environment. These types of studies will provide invaluable insights into the roles of *A. flavus* SMs with respect to fungal virulence, survival, toxigenic potential and modulation of microbial and insect communities, all with the goal of controlling contamination of susceptible crops with *A. flavus* mycotoxins, particularly aflatoxins.

Conflict of interest

The authors have no conflict of interest.

References

1. Gloer JB. Applications of fungal ecology in the search for new bioactive natural products. In: Kubicek CP, Druzhinina IS, eds. *The Mycota, Volume IV*. 2nd ed. New York: Springer-Verlag; 2007:257–283.
2. Horn BW, Moore GG, Carbone I. Sexual reproduction in *Aspergillus flavus*. *Mycologia*. 2009; **101**: 423–429. doi: 10.3852/09-011. PMID: 19537215
3. Kumar P, Mahato DK, Kamle M, Mohanta TK, Kang SG. Aflatoxins: A Global Concern for Food Safety, Human Health and Their Management. *Front Microbiol*. 2017; **7**: 2170. PMID: 28144235
4. Wu F, Liu Y, Bhatnagar D. Cost-effectiveness of aflatoxin control methods: economic incentives. *Toxin Rev*. 2008; **27**: 203–225. doi: 10.1080/15569540802393690.
5. Robens J, Cardwell K. The Costs of Mycotoxin Management to the USA: Management of Aflatoxins in the United States. *J Toxicol Toxin Rev*. 2003; **22**: 139–152. doi: 10.1081/TXR-120024089.
6. Mc Millian WW, Wilson DM, Widstrom NW. Aflatoxin contamination of preharvest corn in Georgia: A six-year study of insect damage and visible *Aspergillus flavus*. *J Environ Qual*. 1985; **14**: 200–202. doi: 10.2134/jeq1985.00472425001400020010x.
7. Nierman WC, Yu J, Fedorova-Abrams ND, et al. Genome sequence of *Aspergillus flavus* NRRL 3357, a strain that causes aflatoxin contamination of food and feed. *Genome Announc*. 2015; **3**: e00168-15. doi: 10.1128/genomeA.00168-15. PMID: 25883274
8. Georgianna DR, Fedorova ND, Burroughs JL, et al. Beyond aflatoxin: four distinct expression patterns and functional roles associated with *Aspergillus flavus* secondary metabolism gene clusters. *Mol Plant Pathol*. 2010; **11**: 213–226. doi: 10.1111/j.1364-3703.2009.00594.x. PMID: 20447271
9. Marui J, Yamane N, Ohashi-Kunihiro S, et al. Kojic acid biosynthesis in Aspergillus oryzae is regulated by a Zn(II)2Cys6 transcriptional activator and induced by kojic acid at the transcriptional level. *J Biosci Bioeng*. 2011; **112**: 40–43. doi: 10.1016/j.jbiosc.2011.03.010. PMID: 21514215
10. Umemura M, Koike H, Nagano N, et al. MIDDAS-M: motif-independent de novo detection of secondary metabolite gene clusters through the integration of genome sequencing and transcriptome data. *PLoS One*. 2013; **8**: e84028. doi: 10.1371/journal.pone.0084028. PMID: 24391870
11. Cole RJ, Schweikert MA, Jarvis BB. *Handbook of Secondary Fungal Metabolites, 3-Volume Set.* Elsevier Science; 2003.
12. Turner WB, Aldridge DC. *Fungal metabolites.* Academic Press; 1983.
13. Vadlapudi V, Borah N, Yellusani KR, et al. *Aspergillus* Secondary Metabolite Database, a resource to understand the Secondary metabolome of *Aspergillus* genus. *Sci Rep*. 2017; **7**: 7325. doi: 10.1038/s41598-017-07436-w. PMID: 28779078
14. Nagano N, Umemura M, Izumikawa M, et al. Class of cyclic ribosomal peptide synthetic genes in filamentous fungi. *Fungal Genet Biol*. 2016; **86**: 58–70. doi: 10.1016/j.fgb.2015.12.010. PMID: 26703898
15. Cary JW, Uka V, Han Z, et al. An Aspergillus flavus secondary metabolic gene cluster containing a hybrid PKS–NRPS is necessary for synthesis of the 2-pyridones, leporins. *Fungal Genet Biol*. 2015; **81**: 88–97. doi: 10.1016/j.fgb.2015.05.010. PMID: 26051490
16. Forseth RR, Amaike S, Schwenk D, et al. Homologous NRPS-like gene clusters mediate redundant small-molecule biosynthesis in *Aspergillus flavus*. *Angew Chem Int Ed*. 2012; **51**: 1–7. PMID: 23281040
17. Umemura M, Nagano N, Koike H, et al. Characterization of the biosynthetic gene cluster for the ribosomally synthesized cyclic peptide ustiloxin B in *Aspergillus flavus*. *Fungal Genet Biol*. 2014; **68**: 23–30. doi: 10.1016/j.fgb.2014.04.011. PMID: 24841822
18. Ehrlich K, Mack B. Comparison of expression of secondary metabolite biosynthesis cluster genes in *Aspergillus flavus, A. parasiticus*, and *A. oryzae*. *Toxins (Basel)*. 2014; **6**: 1916–1928. doi: 10.3390/toxins6061916. PMID: 24960201
19. Strauss J, Reyes-Dominguez Y. Regulation of secondary metabolism by chromatin structure and epigenetic codes. *Fungal Genet Biol*. 2011; **48**: 62–69. doi: 10.1016/j.fgb.2010.07.009. PMID: 20659575

20. Brakhage AA, Schroeckh V. Fungal secondary metabolites – Strategies to activate silent gene clusters. *Fungal Genet Biol*. 2011; **48**: 15–22. doi: 10.1016/j.fgb.2010.04.004. PMID: 20433937
21. Brakhage AA. Regulation of fungal secondary metabolism. *Nat Rev Microbiol*. 2013; **11**: 21–32. doi: 10.1038/nrmicro2916. PMID: 23178386
22. Yu J. Current understanding on aflatoxin biosynthesis and future perspective in reducing aflatoxin contamination. *Toxins (Basel)*. 2012; **4**: 1024–1057. doi: 10.3390/toxins4111024. PMID: 23202305
23. Amaike S, Keller NP. *Aspergillus flavus*. *Annu Rev Phytopathol*. 2011; **49**: 107–133. doi: 10.1146/annurev-phyto-072910-095221. PMID: 21513456
24. Chang PK, Horn BW, Dorner JW. Clustered genes involved in cyclopiazonic acid production are next to the aflatoxin biosynthesis gene cluster in *Aspergillus flavus*. *Fungal Genet Biol*. 2009; **46**: 176–182. doi: 10.1016/j.fgb.2008.11.002. PMID: 19038354
25. Tokuoka M, Seshime Y, Fujii I, Kitamoto K, Takahashi T, Koyama Y. Identification of a novel polyketide synthase–nonribosomal peptide synthetase (PKS–NRPS) gene required for the biosynthesis of cyclopiazonic acid in *Aspergillus oryzae*. *Fungal Genet Biol*. 2008; **45**: 1608–1615. doi: 10.1016/j.fgb.2008.09.006. PMID: 18854220
26. Gilbert MK, Mack BM, Wei Q, Bland JM, Bhatnagar D, Cary JW. RNA sequencing of an *nsdC* mutant reveals global regulation of secondary metabolic gene clusters in *Aspergillus flavus*. *Microbiol Res*. 2016; **182**: 150–161. doi: 10.1016/j.micres.2015.08.007. PMID: 26686623
27. Cary JW, Harris-Coward PY, Ehrlich KC, et al. NsdC and NsdD affect *Aspergillus flavus* morphogenesis and aflatoxin production. *Eukaryot Cell*. 2012; **11**: 1104–1111. doi: 10.1128/EC.00069-12. PMID: 22798394
28. Chang PK, Scharfenstein LL, Li P, Ehrlich KC. *Aspergillus flavus* VelB acts distinctly from VeA in conidiation and may coordinate with FluG to modulate sclerotial production. *Fungal Genet Biol*. 2013; **58-59**: 71–79. doi: 10.1016/j.fgb.2013.08.009. PMID: 23994319
29. Cary JW, Han Z, Yin Y, et al. Transcriptome Analysis of *Aspergillus flavus* Reveals *veA* -Dependent Regulation of Secondary Metabolite Gene Clusters, Including the Novel Aflavarin Cluster. *Eukaryot Cell*. 2015; **14**: 983–997. doi: 10.1128/EC.00092-15. PMID: 26209694
30. Gilbert MK, Mack BM, Payne GA, Bhatnagar D. Use of functional genomics to assess the climate change impact on *Aspergillus flavus* and aflatoxin production. *World Mycotoxin J*. 2016; **9**: 665–672. doi: 10.3920/WMJ2016.2049.
31. Alves PC, Hartmann DO, Núñez O, et al. Transcriptomic and metabolomic profiling of ionic liquid stimuli unveils enhanced secondary metabolism in *Aspergillus nidulans*. *BMC Genomics*. 2016; **17**: 284. PMID: 27072538
32. Chen ZY, Warburton ML, Hawkins L, et al. Production of the 14 kDa trypsin inhibitor protein is important for maize resistance against *Aspergillus flavus* infection/aflatoxin accumulation. *World Mycotoxin J*. 2016; **9**: 215–228. doi: 10.3920/WMJ2015.1890.
33. Brown RL, Chen Z, Menkir A, Cleveland TE. Proteomics to identify resistance factors in corn-a review. *Mycotoxin Res*. 2006; **22**: 22–26. doi: 10.1007/BF02954553. PMID: 23605497
34. Heynen-Genel S, Pache L, Chanda SK, Rosen J. Functional genomic and high-content screening for target discovery and deconvolution. *Expert Opin Drug Discov*. 2012; **7**: 955–968. doi: 10.1517/17460441.2012.711311. PMID: 22860749
35. Yao Z, Petschnigg J, Ketteler R, Stagljar I. Application guide for omics approaches to cell signaling. *Nat Chem Biol*. 2015; **11**: 387–397. doi: 10.1038/nchembio.1809. PMID: 25978996
36. Agrotis A, Ketteler R. A new age in functional genomics using CRISPR/Cas9 in arrayed library screening. *Front Genet*. 2015; **6**: 300. doi: 10.3389/fgene.2015.00300. PMID: 26442115
37. Oliver S, Winson MK, Kell DB, Baganz F. Systematic functional analysis of the yeast genome. *Trends Biotechnol*. 1998; **16**: 373–378. doi: 10.1016/S0167-7799(98)01214-1. PMID: 9744112
38. Pechanova O, Pechan T, Rodriguez JM, Paul Williams W, Brown AE. A two-dimensional proteome map of the aflatoxigenic fungus *Aspergillus flavus*. *Proteomics*. 2013; **13**: 1513–1518. doi: 10.1002/pmic.201100659. PMID: 23457007
39. Fromont-Racine M, Rain JC, Legrain P. Toward a functional analysis of the yeast genome through exhaustive two-hybrid screens. *Nat Genet*. 1997; **16**: 277–282. doi: 10.1038/ng0797-277. PMID: 9207794
40. Panagiotou G, Andersen MR, Grotkjaer T, Regueira TB, Nielsen J, Olsson L. Studies of the production of fungal polyketides in *Aspergillus nidulans* by using systems biology tools. *Appl Environ Microbiol*. 2009; **75**: 2212–2220. doi: 10.1128/AEM.01461-08. PMID: 19168657
41. Saito K, Matsuda F. Metabolomics for functional genomics, systems biology, and biotechnology. *Annu Rev Plant Biol*. 2010; **61**: 463–489. doi: 10.1146/annurev.arplant.043008.092035. PMID: 19152489
42. Cary JW, OBrian GR, Nielsen DM, et al. Elucidation of veA-dependent genes associated with aflatoxin and sclerotial production in *Aspergillus flavus* by functional genomics. *Appl Microbiol Biotechnol*. 2007; **76**: 1107–1118. PMID: 17646985
43. Smith CA, Robertson D, Yates B, et al. The effect of temperature on Natural Antisense Transcript (NAT) expression in *Aspergillus flavus*. *Curr Genet*. 2008; **54**: 241–269. doi: 10.1007/s00294-008-0215-9. PMID: 18813928
44. Wilkinson JR, Yu J, Bland JM, Nierman WC, Bhatnagar D, Cleveland TE. Amino acid supplementation reveals differential regulation of aflatoxin biosynthesis in *Aspergillus flavus* NRRL 3357 and *Aspergillus parasiticus* SRRC 143. *Appl Microbiol Biotechnol*. 2007; **74**: 1308–1319. doi: 10.1007/s00253-006-0768-9. PMID: 17216451
45. Yu J, Cleveland TE, Wilkinson JR, et al. *Aspergillus flavus* expressed sequence tags and microarray as tools in understanding aflatoxin biosynthesis. *Mycotoxin Res*. 2006; **22**: 16–21. doi: 10.1007/BF02954552. PMID: 23605496
46. OBrian GR, Fakhoury AM, Payne GA. Identification of genes differentially expressed during aflatoxin biosynthesis in *Aspergillus flavus* and *Aspergillus parasiticus*. *Fungal Genet Biol*. 2003; **39**: 118–127. doi: 10.1016/S1087-1845(03)00014-8. PMID: 12781670

47. Guo BZ, Yu J, Holbrook CC, Lee RD, Lynch RE. Application of Differential Display RT-PCR and EST/Microarray Technologies to the Analysis of Gene Expression in Response to Drought Stress and Elimination of Aflatoxin Contamination in Corn and Peanut. *J Toxicol Toxin Rev*. 2003; **22**: 287–312. doi: 10.1081/TXR-120024095.
48. Yu J, Ronning CM, Wilkinson JR, et al. Gene profiling for studying the mechanism of aflatoxin biosynthesis in *Aspergillus flavus* and *A. parasiticus. Food Additives & Contaminants.* 2007;24(10):1035–1042.
49. Reverberi M, Punelli M, Scala V, et al. Genotypic and phenotypic versatility of *Aspergillus flavus* during maize exploitation. *PLoS One*. 2013; **8**: e68735. doi: 10.1371/journal.pone.0068735. PMID: 23894339
50. Wilkinson JR, Kale SP, Bhatnagar D, Yu J, Ehrlich KC. Expression profiling of non-aflatoxigenic *Aspergillus parasiticus* mutants obtained by 5-azacytosine treatment or serial mycelial transfer. *Toxins (Basel)*. 2011; **3**: 932–948. doi: 10.3390/toxins3080932. PMID: 22069749
51. Wang H, Lei Y, Yan L, et al. Functional genomic analysis of *Aspergillus flavus* interacting with resistant and susceptible peanut. *Toxins (Basel)*. 2016; **8**: 46. doi: 10.3390/toxins8020046. PMID: 26891328
52. Cary JW, Harris-Coward PY, Ehrlich KC, et al. Functional characterization of a veA-dependent polyketide synthase gene in *Aspergillus flavus* necessary for the synthesis of asparasone, a sclerotium-specific pigment. *Fungal Genet Biol*. 2014; **64** (Supplement C): 25–35. doi: 10.1016/j.fgb.2014.01.001. PMID: 24412484
53. Goodwin S, McPherson JD, McCombie WR. Coming of age: ten years of next-generation sequencing technologies. 2016;17:333.
54. Zhao S, Fung-Leung WP, Bittner A, Ngo K, Liu X. Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cells. *PLoS One*. 2014; **9**: e78644. PMID: 24454679
55. ´t Hoen PAC, Ariyurek Y, Thygesen HH, et al. Deep sequencing-based expression analysis shows major advances in robustness, resolution and inter-lab portability over five microarray platforms. *Nucleic Acids Research*. 2008;36(21):e141-e141.
56. Wang H, Lei Y, Yan L, et al. Deep sequencing analysis of transcriptomes in *Aspergillus flavus* in response to resveratrol. *BMC Microbiol*. 2015; **15**: 182. doi: 10.1186/s12866-015-0513-6. PMID: 26420172
57. Liu SY, Lin JQ, Wu HL, et al. Bisulfite sequencing reveals that *Aspergillus flavus* holds a hollow in DNA methylation. *PLoS One*. 2012; **7**: e30349. doi: 10.1371/journal.pone.0030349. PMID: 22276181
58. Lin JQ, Zhao XX, Zhi QQ, Zhao M, He ZM. Transcriptomic profiling of *Aspergillus flavus* in response to 5-azacytidine. *Fungal Genet Biol*. 2013; **56** (Supplement C): 78–86. doi: 10.1016/j.fgb.2013.04.007. PMID: 23644151
59. Chang PK, Scharfenstein LL, Mack B, Yu J, Ehrlich KC. Transcriptomic profiles of *Aspergillus flavus* CA42, a strain that produces small sclerotia, by decanal treatment and after recovery. *Fungal Genet Biol*. 2014; **68** (Supplement C): 39–47. doi: 10.1016/j.fgb.2014.04.007. PMID: 24780887
60. Fountain JC, Bajaj P, Nayak SN, et al. Responses of *Aspergillus flavus* to Oxidative Stress Are Related to Fungal Development Regulator, Antioxidant Enzyme, and Secondary Metabolite Biosynthetic Gene Expression. *Front Microbiol*. 2016; **7**: 2048. doi: 10.3389/fmicb.2016.02048. PMID: 28066369
61. Medina A, Rodriguez A, Magan N. Effect of climate change on *Aspergillus flavus* and aflatoxin B1 production. *Front Microbiol*. 2014; **5**: 348. doi: 10.3389/fmicb.2014.00348. PMID: 25101060
62. Bai Y, Lan F, Yang W, et al. sRNA profiling in *Aspergillus flavus* reveals differentially expressed miRNA-like RNAs response to water activity and temperature. *Fungal Genet Biol*. 2015; **81** (Supplement C): 113–119. doi: 10.1016/j.fgb.2015.03.004. PMID: 25813270
63. Zhang F, Guo Z, Zhong H, et al. RNA-Seq-based transcriptome analysis of aflatoxigenic *Aspergillus flavus* in response to water activity. *Toxins (Basel)*. 2014; **6**: 3187–3207. doi: 10.3390/toxins6113187. PMID: 25421810
64. Medina A, Gilbert MK, Mack BM, et al. Interactions between water activity and temperature on the *Aspergillus flavus* transcriptome and aflatoxin B_1 production. *Int J Food Microbiol*. 2017; **256**: 36–44. doi: 10.1016/j.ijfoodmicro.2017.05.020. PMID: 28582664
65. Yu J, Fedorova ND, Montalbano BG, et al. Tight control of mycotoxin biosynthesis gene expression in *Aspergillus flavus* by temperature as revealed by RNA-Seq. *FEMS Microbiol Lett*. 2011; **322**: 145–149. doi: 10.1111/j.1574-6968.2011.02345.x. PMID: 21707733
66. Medina A, Schmidt-Heydt M, Rodríguez A, Parra R, Geisen R, Magan N. Impacts of environmental stress on growth, secondary metabolite biosynthetic gene clusters and metabolite production of xerotolerant/xerophilic fungi. *Curr Genet*. 2015; **61**: 325–334. doi: 10.1007/s00294-014-0455-9. PMID: 25381155
67. Medina Á, Rodríguez A, Sultan Y, Magan N. Climate change factors and *Aspergillus flavus* : effects on gene expression, growth and aflatoxin production. *World Mycotoxin J*. 2015; **8**: 171–179.
68. Aoki-Kinoshita KF, Kanehisa M. Gene annotation and pathway mapping in KEGG. *Methods Mol Biol*. 2007; **396**: 71–91. doi: 10.1007/978-1-59745-515-2_6. PMID: 18025687
69. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics*. 2008; **9**: 559. doi: 10.1186/1471-2105-9-559. PMID: 19114008
70. Young MD, Wakefield MJ, Smyth GK, Oshlack A. Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol*. 2010; **11**: R14. doi: 10.1186/gb-2010-11-2-r14. PMID: 20132535
71. Inglis DO, Binkley J, Skrzypek MS, et al. Comprehensive annotation of secondary metabolite biosynthetic genes and gene clusters of *Aspergillus nidulans, A. fumigatus, A. niger* and *A. oryzae*. *BMC Microbiol*. 2013; **13**: 91. doi: 10.1186/1471-2180-13-91. PMID: 23617571
72. Khaldi N, Seifuddin FT, Turner G, et al. SMURF: Genomic mapping of fungal secondary metabolite clusters. *Fungal Genet Biol*. 2010; **47**: 736–741. doi: 10.1016/j.fgb.2010.06.003. PMID: 20554054

73. Andersen MR, Nielsen JB, Klitgaard A, et al. Accurate prediction of secondary metabolite gene clusters in filamentous fungi. *Proc Natl Acad Sci USA*. 2013; **110**: E99–E107. PMID: 23248299
74. Weber T, Kim HU. The secondary metabolite bioinformatics portal: Computational tools to facilitate synthetic biology of secondary metabolite production. *Synthetic and Systems Biotechnology*. 2016; **1**: 69–79. doi: 10.1016/j.synbio.2015.12.002. PMID: 29062930
75. JCVI Smurf − Secondary Metabolite Unique Regions Finder. 2010; http://www.jcvi.org/smurf/index.php. Accessed 10/25/2017.
76. JCVI Storage of all code of the SMURF tool (smurf.jcvi.org). 2015; https://github.com/rsanka/smurf. Accessed 10/25/2017.
77. Blin K, Wolf T, Chevrette MG, et al. antiSMASH 4.0—improvements in chemistry prediction and gene cluster boundary identification. *Nucleic Acids Res*. 2017; **45** (W1): W36–W41. doi: 10.1093/nar/gkx319. PMID: 28460038
78. Medema MH, Blin K, Cimermancic P, et al. antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic Acids Res*. 2011;39(Web Server issue):W339-W346.
79. Weber T, Blin K, Duddela S, et al. antiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Research*. 2015;43(Web Server issue):W237-W243.
80. Umemura M, Koike H, Machida M. Motif-independent de novo detection of secondary metabolite gene clusters—toward identification from filamentous fungi. *Front Microbiol*. 2015; **6**: 371. doi: 10.3389/fmicb.2015.00371. PMID: 25999925
81. Vesth TC, Brandl J, Andersen MR. FunGeneClusterS: Predicting fungal gene clusters from genome and transcriptome data. *Synthetic and Systems Biotechnology*. 2016; **1**: 122–129. doi: 10.1016/j.synbio.2016.01.002. PMID: 29062935
82. Chiang YM, Szewczyk E, Davidson AD, et al. Characterization of the *Aspergillus nidulans* monodictyphenone gene cluster. *Appl Environ Microbiol*. 2010; **76**: 2067–2074. doi: 10.1128/AEM.02187-09. PMID: 20139316
83. Medema MH, Kottmann R, Yilmaz P, et al. Minimum Information about a Biosynthetic Gene cluster. *Nat Chem Biol*. 2015; **11**: 625–631. doi: 10.1038/nchembio.1890. PMID: 26284661
84. Medema MH, Takano E, Breitling R. Detecting sequence homology at the gene cluster level with MultiGeneBlast. *Mol Biol Evol*. 2013; **30**: 1218–1223. doi: 10.1093/molbev/mst025. PMID: 23412913
85. Macheleidt J, Mattern DJ, Fischer J, et al. Regulation and role of fungal secondary metabolites. *Annu Rev Genet*. 2016; **50**: 371–392. doi: 10.1146/annurev-genet-120215-035203. PMID: 27732794
86. Kale SP, Milde L, Trapp MK, Frisvad JC, Keller NP, Bok JW. Requirement of LaeA for secondary metabolism and sclerotial production in *Aspergillus flavus*. *Fungal Genet Biol*. 2008; **45**: 1422–1429. doi: 10.1016/j.fgb.2008.06.009. PMID: 18667168
87. Bok JW, Keller NP. Insight into fungal secondary metabolism from ten years of LaeA research. In: Hoffmeister D, ed. *The Mycota: Biochemistry and Molecular Biology III.* 3rd Edition ed. Cham: Springer International Publishing; 2016:21–29.
88. Oda K, Kobayashi A, Ohashi S, Sano M. *Aspergillus oryzae laeA* regulates kojic acid synthesis genes. *Biosci Biotechnol Biochem*. 2011; **75**: 1832–1834. doi: 10.1271/bbb.110235. PMID: 21897021
89. Calvo AM. The VeA regulatory system and its role in morphological and chemical development in fungi. *Fungal Genet Biol*. 2008; **45**: 1053–1061. doi: 10.1016/j.fgb.2008.03.014. PMID: 18457967
90. Duran RM, Cary JW, Calvo AM. Production of cyclopiazonic acid, aflatrem, and aflatoxin by *Aspergillus flavus* is regulated by *veA*, a gene necessary for sclerotial formation. *Appl Microbiol Biotechnol*. 2006; **73**: 1158–1168. doi: 10.1007/s00253-006-0581-5. PMID: 16988822
91. Kim HR, Chae KS, Han KH, Han DM. The *nsdC* gene encoding a putative C2H2-type transcription factor is a key activator of sexual development in *Aspergillus nidulans*. *Genetics*. 2009; **182**: 771–783. doi: 10.1534/genetics.109.101667. PMID: 19416940
92. Hynes MJ, Murray SL, Duncan A, Khew GS, Davis MA. Regulatory genes controlling fatty acid catabolism and peroxisomal functions in the filamentous fungus *Aspergillus nidulans*. *Eukaryot Cell*. 2006; **5**: 794–805. doi: 10.1128/EC.5.5.794-805.2006. PMID: 16682457
93. Luo X, Affeldt KJ, Keller NP. Characterization of the Far transcription factor family in *Aspergillus flavus*. *G3: Genes|Genomes|Genetics*. 2016;6(10):3269–3281.
94. Chang PK, Scharfenstein LL, Li RW, Arroyo-Manzanares N, De Saeger S, Diana Di Mavungu J. *Aspergillus flavus* aswA, a gene homolog of *Aspergillus nidulans oefC*, regulates sclerotial development and biosynthesis of sclerotium-associated secondary metabolites. *Fungal Genet Biol*. 2017; **104** (Supplement C): 29–37. doi: 10.1016/j.fgb.2017.04.006. PMID: 28442441
95. Cary J, Harris-Coward P, Scharfenstein L, et al. The *Aspergillus flavus* homeobox gene, *hbx1*, is required for development and aflatoxin production. *Toxins (Basel)*. 2017; **9**: 315. doi: 10.3390/toxins9100315. PMID: 29023405
96. Zhuang Z, Lohmar J, Satterlee T, Cary J, Calvo A. The master transcription factor *mtfA* governs aflatoxin production, morphological development and pathogenicity in the fungus *Aspergillus flavus*. *Toxins (Basel)*. 2016; **8**: 29. doi: 10.3390/toxins8010029. PMID: 26805883
97. Satterlee T, Cary JW, Calvo AM. RmtA, a putative arginine methyltransferase, regulates secondary metabolism and development in *Aspergillus flavus*. *PLoS One*. 2016; **11**: e0155575. doi: 10.1371/journal.pone.0155575. PMID: 27213959
98. Lohmar JM, Harris-Coward PY, Cary JW, Dhingra S, Calvo AM. *rtfA*, a putative RNA-Pol II transcription elongation factor gene, is necessary for normal morphological and chemical development in *Aspergillus flavus*. *Appl Microbiol Biotechnol*. 2016; **100**: 5029–5041. doi: 10.1007/s00253-016-7418-7. PMID: 27020290
99. Woloshuk CP, Foutz KR, Brewer JF, Bhatnagar D, Cleveland TE, Payne GA. Molecular characterization of *aflR*, a regulatory locus for aflatoxin biosynthesis. *Appl Environ Microbiol*. 1994; **60**: 2408–2414. PMID: 8074521

100. Yu JH, Butchko RA, Fernandes M, Keller NP, Leonard TJ, Adams TH. Conservation of structure and function of the aflatoxin regulatory gene *aflR* from *Aspergillus nidulans* and *A. flavus*. *Curr Genet*. 1996; **29**: 549–555. PMID: 8662194

101. Flaherty JE, Weaver MA, Payne GA, Woloshuk CPA. A β-glucuronidase reporter gene construct for monitoring aflatoxin biosynthesis in *Aspergillus flavus*. *Appl Environ Microbiol*. 1995; **61**: 2482–2486. PMID: 7618859

102. Fernandes M, Keller NP, Adams TH. Sequence-specific binding by *Aspergillus nidulans* AflR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. *Mol Microbiol*. 1998; **28**: 1355–1365. doi: 10.1046/j.1365-2958.1998.00907.x. PMID: 9680223

103. Ehrlich KC, Montalbano BG, Cary JW. Binding of the C6-zinc cluster protein, AFLR, to the promoters of aflatoxin pathway biosynthesis genes in *Aspergillus parasiticus*. *Gene*. 1999; **230**: 249–257. doi: 10.1016/S0378-1119(99)00075-X. PMID: 10216264

104. Meyers DM, Obrian G, Du WL, Bhatnagar D, Payne GA. Characterization of *aflJ*, a gene required for conversion of pathway intermediates to aflatoxin. *Appl Environ Microbiol*. 1998; **64**: 3713–3717. PMID: 9758789

105. Lim FY, Sanchez JF, Wang CCC, Keller NP. Toward awakening cryptic secondary metabolite gene clusters in filamentous fungi. *Methods Enzymol*. 2012; **517**: 303–324. doi: 10.1016/B978-0-12-404634-4.00015-2. PMID: 23084945

106. Netzker T, Fischer J, Weber J, et al. Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters. *Front Microbiol*. 2015; **6**: 299. doi: 10.3389/fmicb.2015.00299. PMID: 25941517

107. Deepika VB, Murali TS, Satyamoorthy K. Modulation of genetic clusters for synthesis of bioactive molecules in fungal endophytes: A review. *Microbiol Res*. 2016; **182** (Supplement C): 125–140. doi: 10.1016/j.micres.2015.10.009. PMID: 26686621

108. Sobolev VS, Cole RJ, Dorner JW, Horn BW, Harrigan GG, Gloer JB. Isolation and structure elucidation of a new metabolite produced by *Aspergillus parasiticus*. *J Nat Prod*. 1997; **60**: 847–850. doi: 10.1021/np970131m.

109. Zhang S, Monahan BJ, Tkacz JS, Scott B. Indole-diterpene gene cluster from *Aspergillus flavus*. *Appl Environ Microbiol*. 2004; **70**: 6875–6883. doi: 10.1128/AEM.70.11.6875-6883.2004. PMID: 15528556

110. Barrow CJ, Sedlock DM. 1′-(2-Phenyl-ethylene)-ditryptophenaline, a new dimeric diketopiperazine from *Aspergillus flavus*. *J Nat Prod*. 1994; **57**: 1239–1244. doi: 10.1021/np50111a008. PMID: 7528269

111. Terabayashi Y, Sano M, Yamane N, et al. Identification and characterization of genes responsible for biosynthesis of kojic acid, an industrially important compound from *Aspergillus oryzae*. *Fungal Genet Biol*. 2010; **47**: 953–961. doi: 10.1016/j.fgb.2010.08.014. PMID: 20849972

112. Hang L, Liu N, Tang Y. Coordinated and Iterative Enzyme Catalysis in Fungal Polyketide Biosynthesis. *ACS Catal*. 2016; **6**: 5935–5945. doi: 10.1021/acscatal.6b01559. PMID: 28529817

113. Keller NP, Turner G, Bennett JW. Fungal secondary metabolism — from biochemistry to genomics. *Nat Rev Microbiol*. 2005; **3**: 937–947. doi: 10.1038/nrmicro1286. PMID: 16322742

114. Cox RJ, Simpson TJ. Fungal type I polyketide synthases. *Methods Enzymol*. 2009; **459**: 49–78. doi: 10.1016/S0076-6879(09)04603-5. PMID: 19362635

115. Walsh CT, Tang Y. Carbon-based Radicals in C-C bond Formations in Natural Products A. Oxidases B. Oxygenases. In: *Natural Product Biosynthesis: Chemical Logic and Enzymatic Machinery*. London: Royal Society of Chemistry; 2017:457–522.

116. Eisfeld K. Non-Ribosomal Peptide Synthetases of Fungi. In: Anke T, Weber D, eds. *Physiology and Genetics: Selected Basic and Applied Aspects*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2009:305–330.

117. Haynes SW, Ames BD, Gao X, Tang Y, Walsh CT. Unraveling terminal C-domain-mediated condensation in fungal biosynthesis of imidazoindolone metabolites. *Biochemistry*. 2011; **50**: 5668–5679. doi: 10.1021/bi2004922. PMID: 21591693

118. Gao X, Haynes SW, Ames BD, et al. Cyclization of fungal nonribosomal peptides by a terminal condensation-like domain. *Nat Chem Biol*. 2012; **8**: 823–830. doi: 10.1038/nchembio.1047. PMID: 22902615

119. Chang PK, Ehrlich K, Fujii I. Cyclopiazonic acid biosynthesis of Aspergillus flavus and Aspergillus oryzae. *Toxins (Basel)*. 2009; **1**: 74–99. doi: 10.3390/toxins1020074. PMID: 22069533

120. Metzger U, Schall C, Zocher G, et al. The structure of dimethylallyl tryptophan synthase reveals a common architecture of aromatic prenyltransferases in fungi and bacteria. *Proc Natl Acad Sci USA*. 2009; **106**: 14309–14314. doi: 10.1073/pnas.0904897106. PMID: 19706516

121. Xu W, Gavia DJ, Tang Y. Biosynthesis of fungal indole alkaloids. *Nat Prod Rep*. 2014; **31**: 1474–1487. doi: 10.1039/C4NP00073K. PMID: 25180619

122. Saikia S, Nicholson MJ, Young C, Parker EJ, Scott B. The genetic basis for indole-diterpene chemical diversity in filamentous fungi. *Mycol Res*. 2008; **112**: 184–199. doi: 10.1016/j.mycres.2007.06.015. PMID: 18262778

123. Betina V. Thin-layer chromatography of mycotoxins. *J Chromatogr A*. 1985; **334**: 211–276. doi: 10.1016/S0021-9673(00)80272-1. PMID: 2934402

124. Jork H, Funk W, Fischer WR, Wimmer H. *Physical and Chemical Detection Methods: Fundamentals, Reagents I, Volume 1a, Thin-Layer Chromatography: Reagents and Detection Methods*. Wiley-VCH; 1989.

125. Frisvad JC, Thrane U. Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV—VIS spectra (diodearray detection). *J Chromatogr A*. 1987; **404**: 195–214. doi: 10.1016/S0021-9673(01)86850-3. PMID: 3680432

126. Smedsgaard J. Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. *J Chromatogr A*. 1997; **760**: 264–270. doi: 10.1016/S0021-9673(96)00803-5. PMID: 9062989

127. Nielsen K, Smedsgaard J. Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for dereplication by standardised liquid chromatography–UV–mass spectrometry methodology. *J Chromatogr A*. 2003; **1002**: 111–136. doi: 10.1016/S0021-9673(03)00490-4. PMID: 12885084

128. Sulyok M, Krska R, Schuhmacher R. A liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application to semi-quantitative screening of moldy food samples. *Anal Bioanal Chem*. 2007; **389**: 1505–1523. doi: 10.1007/s00216-007-1542-2. PMID: 17874237

129. Klitgaard A, Iversen A, Andersen MR, Larsen TO, Frisvad JC, Nielsen KF. Aggressive dereplication using UHPLC–DAD–QTOF: screening extracts for up to 3000 fungal secondary metabolites. *Anal Bioanal Chem*. 2014; **406**: 1933–1943. doi: 10.1007/s00216-013-7582-x. PMID: 24442010

130. Nielsen KF, Månsson M, Rank C, Frisvad JC, Larsen TO. Dereplication of microbial natural products by LC-DAD-TOFMS. *J Nat Prod*. 2011; **74**: 2338–2348. doi: 10.1021/np200254t. PMID: 22026385

131. Rank C, Klejnstrup ML, Petersen LM, et al. Comparative Chemistry of Aspergillus oryzae (RIB40) and A. flavus (NRRL 3357). *Metabolites*. 2012; **2**: 39–56. doi: 10.3390/metabo2010039. PMID: 24957367

132. Malysheva SV, Arroyo-Manzanares N, Cary JW, et al. Identification of novel metabolites from *Aspergillus flavus* by high resolution and multiple stage mass spectrometry. *Food Additives & Contaminants: Part A*. 2014; **31**: 111–120. doi: 10.1080/19440049.2013.859743. PMID: 24405210

133. Arroyo-Manzanares N, Diana Di Mavungu J, Uka V, et al. Use of UHPLC high-resolution Orbitrap mass spectrometry to investigate the genes involved in the production of secondary metabolites in *Aspergillus flavus*. *Food Additives & Contaminants: Part A*. 2015; **32**: 1656–1673. doi: 10.1080/19440049.2015.1071499. PMID: 26278397

134. Reynolds WF, Mazzola EP. Nuclear Magnetic Resonance in the Structural Elucidation of Natural Products. In: Kinghorn AD, Falk H, Kobayashi J, eds. *Progress in the Chemistry of Organic Natural Products 100.* Cham: Springer International Publishing; 2015:223–309.

135. Forseth RR, Schroeder FC. NMR-spectroscopic analysis of mixtures: from structure to function. *Curr Opin Chem Biol*. 2011; **15**: 38–47. doi: 10.1016/j.cbpa.2010.10.010. PMID: 21071261

136. Robinette SL, Brüschweiler R, Schroeder FC, Edison AS. NMR in metabolomics and natural products research: two sides of the same coin. *Acc Chem Res*. 2012; **45**: 288–297. doi: 10.1021/ar2001606. PMID: 21888316

137. Forseth RR, Schroeder FC. Correlating Secondary Metabolite Production with Genetic Changes Using Differential Analysis of 2D NMR Spectra. In: Keller NP, Turner G, eds. *Fungal Secondary Metabolism: Methods and Protocols.* Totowa, NJ: Humana Press; 2012:207–219.

138. Schroeder FC, Gibson DM, Churchill ACL, et al. Differential analysis of 2D NMR spectra: new natural products from a pilot-scale fungal extract library. *Angew Chem Int Ed*. 2007; **46**: 901–904. doi: 10.1002/anie.200603821. PMID: 17183517

139. Forseth RR, Fox EM, Chung D, Howlett BJ, Keller NP, Schroeder FC. Identification of cryptic products of the gliotoxin gene cluster using NMR-based comparative metabolomics and a model for gliotoxin biosynthesis. *J Am Chem Soc*. 2011; **133**: 9678–9681. doi: 10.1021/ja2029987. PMID: 21612254

140. Nicholson MJ, Koulman A, Monahan BJ, Pritchard BL, Payne GA, Scott B. Identification of two aflatrem biosynthesis gene loci in *Aspergillus flavus* and metabolic engineering of *Penicillium paxilli* to elucidate their function. *Appl Environ Microbiol*. 2009; **75**: 7469–7481. doi: 10.1128/AEM.02146-08. PMID: 19801473

141. TePaske MR, Gloer JB, Wicklow DT, Dowd PF, Leporin A. Leporin A: an antiinsectan N-alkoxypyridone from the sclerotia of *Aspergillus leporis*. *Tetrahedron Lett*. 1991; **32**: 5687–5690. doi: 10.1016/S0040-4039(00)93530-5.

142. Zhang C, Jin L, Mondie B, et al. Leporin B: a novel hexokinase II gene inducing agent from an unidentified fungus. *Bioorg Med Chem Lett*. 2003; **13**: 1433–1435. doi: 10.1016/S0960-894X(03)00153-7. PMID: 12668006

143. Ohashi M, Liu F, Hai Y, et al. SAM-dependent enzyme-catalysed pericyclic reactions in natural product biosynthesis. *Nature*. 2017; **549**: 502–506. doi: 10.1038/nature23882. PMID: 28902839

144. Aguirre J, Ríos-Momberg M, Hewitt D, Hansberg W. Reactive oxygen species and development in microbial eukaryotes. *Trends Microbiol*. 2005; **13**: 111–118. doi: 10.1016/j.tim.2005.01.007. PMID: 15737729

145. Grintzalis K, Vernardis SI, Klapa MI, Georgiou CD. Role of oxidative stress in Sclerotial differentiation and aflatoxin B1 biosynthesis in *Aspergillus flavus*. *Appl Environ Microbiol*. 2014; **80**: 5561–5571. doi: 10.1128/AEM.01282-14. PMID: 25002424

146. TePaske MR, Gloer JB, Wicklow DT, Dowd PF. Aflavarin and beta-aflatrem: new anti-insectan metabolites from the sclerotia of *Aspergillus flavus*. *J Nat Prod*. 1992; **55**: 1080–1086. doi: 10.1021/np50086a008.

147. Bok JW, Keller NP. LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryot Cell*. 2004; **3**: 527–535. doi: 10.1128/EC.3.2.527-535.2004. PMID: 15075281

148. Ehmann DE, Gehring AM, Walsh CT. Lysine biosynthesis in *Saccharomyces cerevisiae*: mechanism of α-aminoadipate reductase (Lys2) involves posttranslational phosphopantetheinylation by Lys5. *Biochemistry*. 1999; **38**: 6171–6177. doi: 10.1021/bi9829940. PMID: 10320345

149. Saikia S, Parker EJ, Koulman A, Scott B. Four gene products are required for the fungal synthesis of the indole-diterpene, paspaline. *FEBS Lett*. 2006; **580**: 1625–1630. doi: 10.1016/j.febslet.2006.02.008. PMID: 16494875

150. Tagami K, Minami A, Fujii R, et al. Rapid reconstitution of biosynthetic machinery for fungal metabolites in *Aspergillus oryzae*: total biosynthesis of aflatrem. *ChemBioChem*. 2014; **15**: 2076–2080. doi: 10.1002/cbic.201402195. PMID: 25087641

151. Tang MC, Lin HC, Li D, et al. Discovery of unclustered fungal indole diterpene biosynthetic pathways through combinatorial pathway reassembly in engineered yeast. *J Am Chem Soc*. 2015; **137**: 13724–13727. doi: 10.1021/jacs.5b06108. PMID: 26469304

152. Gloer JB, TePaske MR, Sima JS, Wicklow DT, Dowd PF. Antiinsectan aflavinine derivatives from the sclerotia of *Aspergillus flavus*. *J Org Chem*. 1988; **53**: 5457–5460. doi: 10.1021/jo00258a011.

153. Gallagher RT, Wilson BJ. Aflatrem, the tremorgenic mycotoxin from *aspergillus flavus*. *Mycopathologia*. 1979; **66**: 183–185. doi: 10.1007/BF00683969. PMID: 108599

154. Koiso Y, Li YIN, Iwasaki S, et al. Ustiloxins, antimitotic cydic peptides from false smut balls on rice panicles caused by Ustilaginoidea virens. *J Antibiot (Tokyo)*. 1994; **47**: 765–773. doi: 10.7164/antibiotics.47.765. PMID: 8071121

155. Koiso Y, Morisaki N, Yamashita Y, et al. Isolation and structure of an antimitotic cyclic peptide, ustiloxin F: chemical interrelation with a homologous peptide, ustiloxin B. *J Antibiot (Tokyo)*. 1998; **51**: 418–422. doi: 10.7164/antibiotics.51.418. PMID: 9630863

156. Yoshimi A, Umemura M, Nagano N, Koike H, Machida M, Abe K. Expression of *ustR* and the Golgi protease KexB are required for ustiloxin B biosynthesis in *Aspergillus oryzae*. *AMB Express*. 2016; **6**: 9. doi: 10.1186/s13568-016-0181-4. PMID: 26842395

157. Saruwatari T, Yagishita F, Mino T, Noguchi H, Hotta K, Watanabe K. Cytochrome P450 as dimerization catalyst in diketopiperazine alkaloid biosynthesis. *ChemBioChem*. 2014; **15**: 656–659. doi: 10.1002/cbic.201300751. PMID: 24677498

158. Sano M. *Aspergillus oryzae nrtA* affects kojic acid production. *Biosci Biotechnol Biochem*. 2016; **80**: 1776–1780. doi: 10.1080/09168451.2016.1176517. PMID: 27108780

159. Dowd PF. Synergism of aflatoxin B1 toxicity with the co-occurring fungal metabolite kojic acid to two caterpillars. *Entomol Exp Appl*. 1988; **47**: 69–71. doi: 10.1111/j.1570-7458.1988.tb02283.x.