Final Report "Metabolic profiling in *Aspergillus flavus* to determine gene function"

(March, 2014-August, 2014)

Location

Laboratory of Food Analysis	Post Doc	Dr. Zheng Han
Faculty of Pharmaceutical Sciences	Promoter	Prof. Dr. Sarah De Saeger
Ghent University	Co-promoter	Dr. José Diana Di Mavungu

ABBREVIATIONS

AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
Aspergillus flavus	*A. flavus*
DTP	Ditryptophenaline
ESI^+	Positive electrospray ionization
ESI^-	Negative electrospray ionization
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NRPSs	Non-ribosomal peptide synthases

EXECUTIVE SUMMARY

Genes for secondary metabolites production are arranged in clusters. Analysis of the *A. flavus* genome revealed 55 putative secondary metabolites gene clusters; the function of most of these is still unknown. In this project, the function of selected *A. flavus* secondary metabolites gene clusters was investigated by gene knockout/overexpression and comparative metabolomics. The present report focuses on two gene clusters, i.e., clusters 11 and 23, which resulted in more useful preliminary data than the initially selected clusters 21 and 34, and have therefore been more extensively studied. Gene overexpression is illustrated using cluster 23 Zn2Cys6 transcription factor, while gene knockout is highlighted using different genes in cluster 11.

Work package (WP) 1. Creating *A. flavus* that overexpress the putative transcription factors (TF) controlling selected biosynthetic gene clusters

An *Aspergillus flavus* CA14 ku70, niaD^{-}, pyrG^{-}, ptrA^{-} parental strain (referred to as CA14 ku70) was used as host for transformation. Three over-expressing mutants, i.e., AFLA_066900 (CA14 ptrA^{+}, orange colony pigmentation) isolates, were obtained. Following polymerase chain reaction (PCR) amplification of *A. flavus* CA14 genomic DNA a PCR product of the expected size of 2537 base pair (bp) for AFLA_066900 was obtained and subsequently subcloned into TOPO pCR2.1 (Life Technologies) and verified by DNA sequencing.

WP 2. Creating knockout mutants for the backbone genes

We created *A. flavus* mutants in which the backbone gene for the non-ribosomal peptide synthase (*nrps)* is disrupted by insertional mutagenesis. Knockout of the genes AFLA_023020 (NRPS-encoding gene), AFLA_023010 (a possible methyltransferase/ desaturase), AFLA_023030 (a cytochrome P450 monooxygenase) and AFLA_023040, a Zn2Cys6 transcription factor were prepared in *A. flavus* CA14 ku70- by insertional mutagenesis using the *A. oryzae* ptrA resistance gene from pTRI.

WP 3. Extraction and concentration of metabolites

An untargeted metabolic profiling was applied in the present research due to the unknown solubility of the various metabolites. Therefore, a sample preparation procedure that was as simple and universal as possible was developed. In the present project, several different extraction and clean-up procedures were optimized and compared. Finally, a robust sample pretreatment method was established, which could be used for the extraction of the various metabolites in different matrices.

WP 4. Metabolite profiling and identification

First, after optimization of the mobile-phase, the gradient elution program, the column, the ionization mode and the MS/MS parameters, the methods using UHPLC-Orbitrap-HRMS and LC-Ion-Trap were established for identification of the unknown compounds. The total run time for each method was no more than 30 minites with nice peak shape and satisfactory separation efficiency.

Second, according to literature, a database including a total of 57 known *A. flavus* biosynthesis products and intermediates was established in the present project.

Finally, the gene cluster 11 knockout/overexpressing mutants, as well as the wide-type strains were analyzed by the established UHPLC-Orbitrap-HRMS and LC-Ion-Trap methods. The metabolites were searched from the database and compared to determine the function of this gene cluster in *A. flavus.* Ditryptophenaline (DTP), which has received attention for its potential pharmaceutical use as a substance P antagonist, was found to be controlled by gene cluster 11. Although DTP is structurally different from the LnaA and LnaB metabolites, given its activity as a substance P antagonist, it is entirely possible that it could serve to mediate signal transmission in *A. flavus*.

Based on the above descriptions, it could be obviously seen that all the work packages (1-4) have been performed, and the objectives set in the project have been achieved.

BACKGROUND

Fungal metabolomics research has recently received much attention mainly for studies in *Aspergillus nidulans* and *Aspergillus fumigatus*. In these studies, silent biosynthesis genes can be turned on using a variety of approaches including: overexpressing the transcription factor that controls their expression, growing the fungi in more 'natural' situations, removing chromatin-modulating proteins that prevent gene activity; placing the biosynthesis gene clusters in heterologous organisms. However, the products of many potential metabolite biosynthetic gene clusters are still unknown. In *A. flavus* and *A. parasiticus*, the only gene clusters so far identified have been the aflatoxin (Yu, Bhatnagar & Cleveland, 2004), cyclopiazonic acid (Chang, Ehrlich & Fujii, 2009) and aflatrem (Nicholson, et al., 2009) biosynthesis gene clusters as well as some pigment producing clusters . In the latter the metabolite precursors have not been identified in most cases. About 20 other metabolites have been identified from *A. flavus* and *A. parasiticus* while precursor metabolites to these are still not known. In other fungi, while some gene clusters have successfully been associated with production of known metabolites, most potential biosynthesis gene clusters have not yet been assigned a function. More importantly, some metabolite gene clusters are silent and no metabolite has been identified for the cluster. This genome mining promises to be a powerful approach to identify new and potentially agriculturally and medically important fungal metabolites.

Analysis of genomes of fungi has allowed the prediction of gene clusters involved in biosynthesis of numerous secondary metabolites, some of which are of pharmaceutical or agrochemical value while others are toxic and render contaminated foods unmarketable (Reddy, *et al.*, 2010). An estimation of the potential of the *A. flavus* genome to produce secondary metabolites formed from non-ribosomal peptide synthases (NRPSs), polyketide synthases and prenyl synthases identified 55 different gene clusters (Georgianna, *et al.*, 2010-Khaldi, *et al.*, 2010). Of these, 29 contain genes predicted to encode non-ribosomal peptide synthases as the core (backbone) gene involved in metabolite biosynthesis. The metabolites associated with a biosynthesis gene cluster are known for only three of these clusters (Chang, *et al.*, 2009-Forseth, *et al.*, 2013). We now show that the NRPS in cluster

11 is involved in the biosynthesis of the indole-diketopiperazine-derived metabolite ditryptophenaline (DTP) (Fig. 1).

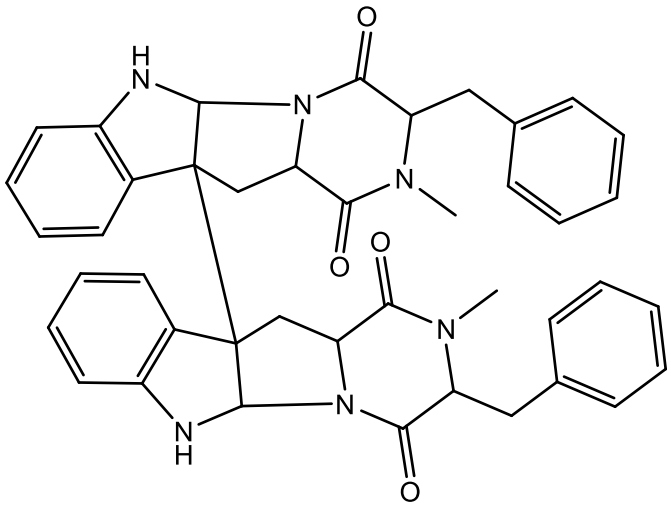

Fig. 1. Structure of ditryptophenaline

DTP is a member of a large family of non-ribosomal peptides, including some whose biosynthetic gene clusters have previously been established (Scharf*, et al.,* 2013). It is distinguished from other indole-diketopiperazines by possession of a 3a,3a'-bispyrrolidinoindoline ring. Compounds with this type of ring structure, the hexahydropyrrolo[2,3-b]indole family of metabolites, are found ubiquitously in nature, but only a few are fused to a diketopiperazine ring. These compounds have a wide range of biological activities ranging from antitumor, antimicrobial, topoisomerase inhibitor, and neurokinin antagonist activities (Cornacchia*, et al.*, 2012-Lu & Lu, 2010). Their specific role in fungi and their biosyntheses have not been elucidated. DTP was first isolated in 1977 (Springer*, et al.*, 1977), and several chemical syntheses of this and analogues have been reported (Movassaghi*, et al.*, 2008-Overman & Paone, 2001). DTP has specific activity as an antagonist of Substance P, an undecapeptide neurotransmitter and neuromodulator that is an essential part of the tachykinin neuropeptide family of peptides (Barrow & Sedlock, 1994).

OBJECTIVES

In *A. flavus*, a total of 55 putative secondary metabolite gene clusters have been predicted using new software named Secondary Metabolite Unique Regions Finder (SMURF) (http://www.jcvi.org/smurf) (Khaldi et al., 2010). As mentioned above, the function for gene clusters and precursor metabolites so far has been established only for aflatoxin, cyclopiazonic acid and aflatrem biosynthesis gene clusters. The other clusters potentially could make metabolites that might be agriculturally or medically important. The major purposes of the proposed research are: (1) to create *A. flavus* mutants that overexpress the putative transcription factors (TF) controlling selected biosynthetic gene clusters (WP 1); (2) to create knockout mutants for the backbone genes (WP2); (3) to establish a general extraction method which can be used for extraction of various metabolites in different matrices (WP3); (4) to investigate the metabolite profiling of the mutants and wide-type strains in order to determine the function of typical gene cluster in *A. flavus* (WP4).

Methodology

Materials and reagents

Methanol and acetonitrile were liquid chromatography-mass spectrometry (LC-MS) grade. Water was purified by a Milli-Q system (Millipore, Brussels, Belgium). All the other regents were LC-MS/MS or HPLC grade.

Yeast, peptone, corn/steep, dextrose, sucrose, $NaNO_3$, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, KCl, $FeSO_4 \cdot 7H_2O$, agar, uracil and $(NH_4)_2SO_4$ were from Sigma-Aldrich (Saint Louis, MO, USA).

Preparation of the WATM medium

Accurately weighed solid portions of different reagents: yeast (2 g), peptone (3 g), corn/steep (5 g), dextrose (2 g), sucrose (30 g), $NaNO_3$(2 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KCl (0.2 g), $FeSO_4 \cdot 7H_2O$ (0.18 g), agar (15 g), uracil (2 g) and $(NH_4)_2SO_4$ (1.32 g) were dissolved into 1 L distilled water. The medium was sterilized and about 20 mL of the medium was poured into one petri dish, and then put aside in the laminar flow until the medium turned to be solid, and ready for inoculation.

Creating overexpressing mutants

An *Aspergillus flavus* CA14 ku70, $niaD^{-}$, $pyrG^{-}$, $ptrA^{-}$ parental strain (referred to as CA14 ku70) was used as host for transformation. Three over-expressing mutants, i.e., AFLA_066900 (CA14 $ptrA^{+}$, orange colony pigmentation) isolates, were obtained following transformation of the parental strain with the AFLA_066900 overexpression vector pPTRI-gpd-AFLA_066900-trpC [pyrithiamine (ptrA) selection] that placed the AFLA_066900 gene under control of the constitutive glyceraldehyde-3-phosphate (gpd) promoter of A. nidulans in the vector pPTRI. To construct the *A. flavus* CA14 pPTRI-gpd-AFLA_066900-trpC overexpression plasmid the AFLA_066900 ORF was amplified by polymerase chain reaction (PCR) using Platinum® Pfx polymerase (Life Technologies, Carlsbad, CA). Following PCR amplification of *A. flavus* CA14 genomic DNA a PCR product of the expected size of 2537 base pair (bp) for AFLA_066900 was obtained and

subsequently subcloned into TOPO pCR2.1 (Life Technologies) and verified by DNA sequencing. The AFLA_066900 coding region was released from the TOPO vector using NotI-RsrII digestion and subcloned into the analogous restriction digested pPTRI vector to generate pPTRI-gpd-AFLA_066900-trpC.

Creating knockout mutants for the backbone genes

The genes in cluster 11 have been identified by SMURF as AFLA_023000 to AFLA_023080 (Khaldi, *et al.*, 2010) (Table 1). Knockout of the genes AFLA_023020 the (NRPS-encoding gene), AFLA_023010 (a possible methyltransferase/desaturase); AFLA_023030 (a cytochrome P450 monooxygenase) and AFLA_023040, a Zn_2Cys_6 transcription factor were prepared in *A. flavus CA14 ku70*$^-$ by insertional mutagenesis according to the method of Szewczyk, et al (Szewczyk, *et al.*, 2008) using the *Aspergillus oryzae ptrA* resistance gene from pTRI (Takara) for selection. Selection was on Czapek's agar plates containing 0.1 µg/ml pyrithiamine (Sigma). Transformation was as previously described (Chang, *et al.*, 2012).

Table 1 Genes identified by SMURF in cluster 11

A. flavus	Protein	Protein length	Nt length	AF3357	*A. oryzae*	AF70	*A. para*	3357
				RNAseq-RPKM				Microarray[a]
AFLA_023000	Ankyrin domain protein	339	1069	6.1	3.8	9.2	0.0	-17.5
AFLA_023010	GA4 desaturase family protein	228	996	32.2	15.0	4.9	5.1	-9.5
AFLA_023020	NRPS-like enzyme, putative	1022	3066	0.2	0.0	6.0	2.7	-15.4
AFLA_023030	Cytochrome P450 family protein	514	1660	0.0	0.7	4.6	3.0	-9.2
AFLA_023040	Fungal specific transcription factor domain containing protein	703	2119	4.6	4.6	4.6	19.0	-4.5
AFLA_023050	Major Facilitator	443	1502	2.8	6.2	6.5	3.2	-24.4

AFLA_023070	Superfamily protein RGS superfamily domain protein	524	1798	80.4	#N/A	#N/A	#N/A	0.0
AFLA_023080	TmpA, ferridoxin reductase domain	472	1416	25.6	338.4	10.2	17.8	-7.1

[a]ΔlaeAmutant/wild type

Extraction

The medium in one plate was brought into one plastic tube (50 mL), and extracted with 30 mL of methanol-dichloromethane-ethyl acetate (v/v/v, 1/2/3) containing 1% formic acid (v/v) by shaking for 60 min. The extraction was collected and divided into 5 tubes, evaporated to dryness by nitrogen gas, and the residues were re-dissolved by methanol-acetonitrile-water (30/30/40, v/v/v).

Instruments

UHPLC-Orbitrap-HRMS Analysis

UHPLC-Exactive^{TM} Benchtop Orbitrap mass spectrometer (Thermo Fisher Scientific, San José, CA, USA) analysis in the full scan mode (*m/z*, 100–1000) was utilized for the metabolic profiling study. Chromatographic separation was achieved on a Zorbax Eclipse Plus C_{18} column (100 mm × 2.1 mm, 1.8 μm) at 30 °C, with a mobile phase flow rate of 0.4 mL min^{-1}. The mobile phase consisted of (A) water/methanol (5/95, v/v) containing 0.1% formic acid and 10 mM ammonium formate and (B) water/methanol (95/5, v/v) containing 0.1% formic acid and 10 mM ammonium formate. A linear gradient elution program was applied as follows: 0 min 0% B, 0.5 min 0% B, 20 min 99.1% B, 21 min 99.1% B, 24 min 0% B and hold on for a further 4 min for re-equilibration, giving a total run time of 28 min. The injection volume was 5.0 μL. The mass spectrometer was operated both in $HESI^+$ and in $HESI^-$. The following settings were used: spray voltage, 4.5 kV; capillary temperature, 250 °C; heater temperature, 250 °C; sheath gas flow, 45 a.u.; auxiliary gas, 10 a.u; sweep

gas, 2 a.u., resolution 100000 FWHM at 1 Hz (1 scan per second). The automatic gain control (AGC) target was set at high dynamic range ($3e^6$), and the maximum injection time was 20 ms. Initial instrument calibration was achieved by infusing calibration mixtures (Thermo Fisher Scientific) for positive and negative ion modes. The positive calibration mixture included caffeine, Met-Arg-Phe-Ala acetate salt (MRFA) and Ultramark 1621®, while the negative calibration solution comprised sodium dodecyl sulfate, sodium taurocholate and Ultramark 1621®. These compounds were dissolved in a mixture of acetonitrile, water and methanol, and both mixtures were infused with a Chemyx Fusion 100 syringe pump (Thermo Fisher Scientific). Data were acquired and processed by Xcalibur 2.1 and Sieve 2.0 software (Thermo Fisher Scientific, Brookfield, Los Angeles, California, USA).

LC-Ion-Trap Analysis

HPLC-ion-trap-MS system (Thermo Fisher Scientific) was used for the fragments analysis of the targeted analytes. The column used was an X-bridge C18 column (3.5 μm, 2.1 × 150 mm), supplied by Waters (Milford, MA, USA). The mobile phase consisted of (A) water/methanol (5/95, v/v) containing 0.1% formic acid and 10 mM ammonium formate and (B) water/methanol (95/5, v/v) containing 0.1% formic acid and 10 mM ammonium formate. The linear gradient elution program for LC-Ion-trap analysis was: 0–1 min B = 50%, 1–13 min B = 50%–97%, 13–18 min B = 97%, 18–19 min B = 97%–50%, and hold on for a further 6 min for re-equilibration, giving a total run time of 25 min. The mass spectrometer was operated both in $HESI^+$ and in $HESI^-$ with the following settings: source voltage of 5 kV, capillary temperature of 250 °C; heater temperature of 175 °C; sheath gas flow of 45 a.u.; aux gas of 10 a.u. The Xcalibur 2.0.7 software (Thermo Scientific) was used for instrument control, data acquisition and processing.

RESULTS and DISCUSSION

Optimization of the extraction and concentration method

Samples of different fungi including BN9_nsdDKO_nr28, BN9_wt_ nr60, and ANI 3157 A nomius were selected for comparison of different extraction methods in the present study:

Method 1 was established according to the methods described by Andersen, et al., 2011 and Smedsgaard, 1997 with minor revisions: The medium in one plate was brought into one plastic tube (50 mL), and extracted with 30 mL of methanol-dichloromethane-ethyl acetate (v/v/v, 1/2/3) containing 1% formic acid (v/v) by shaking for 60 min. An aliquot of extraction (20 mL) was collected and divided into 5 tubes, evaporated to dryness by nitrogen gas, and the residues were re-dissolved by methanol-acetonitrile-water (30/30/40, v/v/v).

Method 2 was established according to the methods described by Frisvad & Thrane, 1987 with minor revisions: The medium in one plate was brought into one plastic tube (50 mL), and firstly extracted with 30 mL of methanol- dichloromethane (1:1) by shaking for 60 min. The extraction was collected in another plastic tube. Then, the residues were extracted with 30 mL of ethyl acetate-acetone (1:1) containing 1% formic acid by shaking for 60 min. Aliquots of extractions (20 mL+20 mL) were collected and divided into 5 tubes. The mixture was evaporated to dryness by nitrogen gas, and re-dissolved by methanol-acetonitrile-water (30/30/40, v/v/v).

Method 3 was established according to the methods described by Ren, et al., 2007 and Han, et al., 2012 with minor revisions: The medium in one plate was brought into one plastic tube (50 mL), and extracted with 30 mL of acetonitrile/water (84/16, v/v) by shaking for 60 min. An aliquot of extraction (20 mL) was collected and divided into 5 tubes. The solution was evaporated to dryness by nitrogen gas, and re-dissolved by methanol-acetonitrile-water (30/30/40, v/v/v).

Method 4 as established according to the methods described by Sulyok, et al., 2006, 2007: The medium in one plate was brought into one plastic tube (50 mL), and extracted

with 30 mL of acetonitrile/water/acetic acid (79:20:1, v/v/v) by shaking for 60 min. An aliquot of extraction (20 mL) was collected and divided into 5 tubes. The solution was evaporated to dryness by nitrogen gas, and re-dissolved by methanol-acetonitrile-water (30/30/40, v/v/v).

Method 5: The medium in one plate was brought into one plastic tube (50 mL), and extracted with 30 mL of acetone by shaking for 60 min. An aliquot of extraction (20 mL) was collected and divided into 5 tubes. The solution was evaporated to dryness by nitrogen gas, and re-dissolved by methanol-acetonitrile-water (30/30/40, v/v/v).

Method 6: The medium in one plate was brought into one plastic tube (50 mL), and extracted with 30 mL of acetone by shaking for 60 min. The extraction was collected in another tube and 5 mL methylene chloride and 15 mL water were added. The mixture was shaken for 10 min and then centrifuged at 2000 rpm for 5min. The layer of methylene chloride was collected and divided into 5 tubes. The solution was evaporated to dryness by nitrogen gas, and re-dissolved by methanol-acetonitrile-water (30/30/40, v/v/v).

For the metabolic study, it is difficult to set an assessment criterion for comparison of different extraction methods. In the present study, since most of the interesting metabolites belong to aflatoxins or the compounds with the same parent molecular, the peak areas of AFB1, AFB2, AFG1 and AFG2 after extraction were utilized for the evaluation of different methods. As shown in Table 2, in most cases, the extraction efficiency of method 1 was higher than that of methods 2, 3, 4, 5 and 6. In addition, method 1 would constitute a simpler and more economic procedure. For example, it took more than 2 h to dry the sample solution when the other methods were used, but no more than 20 min if method 1 was selected. Therefore, method 1 was selected as the extraction method for the present study.

Table 2 Comparison of different extraction methods (responses)

Samples	Methods	AFB1	AFB2	AFG1	AFG2
ANI 3157 A nomius	1	144414315	68052167	2819779798	1141977410
	2	99843159	3682244	1890386135	52865173
	3	115130372	4065804	1794111688	37388630
	4	135369346	15771524	2592466264	88126489
	5	134157373	8366190	2202497879	78301392
	6	321330	877840	2458144	67546

BN9_wt_ nr60	1	143323295	25570918	2594440196	87803134
	2	222225124	23158765	2918596094	96514316
	3	201650337	25799938	2681374690	91059811
	4	172787329	27079042	2309545137	73025217
	5	237295477	15869785	2613358061	82838971
	6	170406	n.d.	2024701	82473
BN9_nsdDKO_nr28	1	2299153676	553244484	79699282	13885312
	2	2670285871	477216532	108227690	1905478
	3	2159680246	312549113	64313124	3215434
	4	2128316093	315351438	50959260	3624556
	5	2258661774	335759911	63204944	4179996
	6	2686167964	454126296	56546884	3323177

Phenotype of A. flavus wide-type and cluster 23 afla_066900 overexpressed strains

The wild type (WT) and cluster 23 over-expressed (OE) strains of *A. flavus* were grown under the same conditions. The growth of the WT strain was much faster over the course of the first five days. Therefore, the over-expression of cluster 23 seemed to have a negative effect on growth, especially at the early stage of culture development. In addition, the WT strain produced a thicker layer of conidiophores compared to that of the OE strain. The edges of the fungal colonies were also different; a sharp clear white edge could be observed in the WT colonies and a larger light-red colored edge was found in the OE colonies (Fig. 2). These differences in growth and phenotype of the WT and the OE strains of *A. flavus* were consistent across the corresponding replicate cultures.

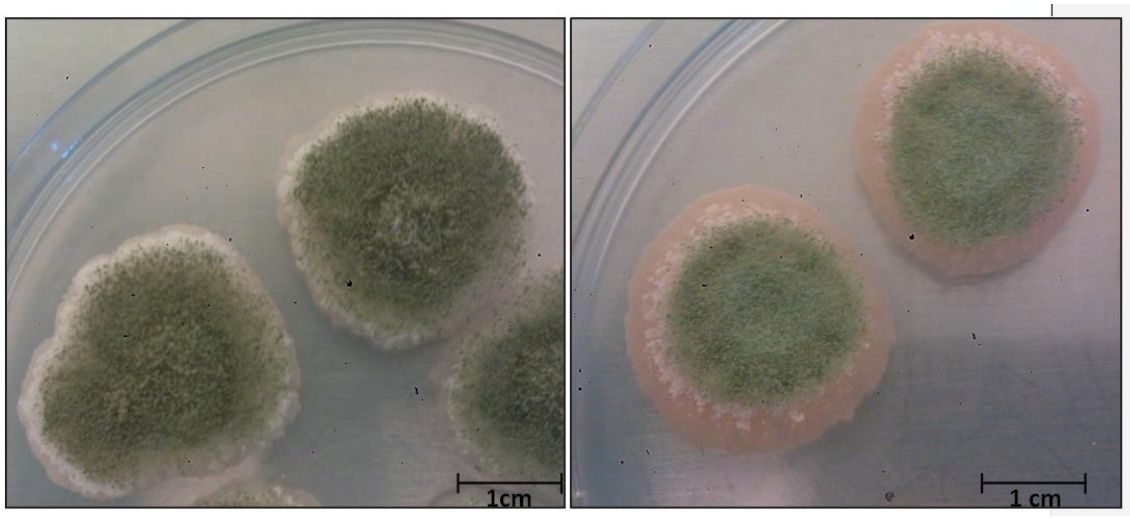

Fig. 2. Clusters of the wide-type (left) and cluster 23 overexpressed (right) A. flavus strains

Data analysis and characterization of the gene cluster 11 for secondary metabolites production

In *A. flavus* the cluster 11 NRPS (AFLA_023020) (Table 1) has similar size (1021aa) and domain structures (A-pp-SDR_e1) to those of 9 other NRPSs identified among the 55 potential gene clusters involved in secondary metabolite production. Of these, microarray (Georgianna, *et al.*, 2010) (Fedorova, personal communication) and RNAseq studies (Table 1) suggested that the cluster 11 NRPS gene, while poorly expressed in liquid minimal salts media, is expressed well on complex agar media (WATM). Blast search of the NCBI fungi database with the cluster 11 NRPS protein revealed that the closest identity was to a gramicidin-S-synthetase (39%ID, 58% pos aa). In the *A. flavus* genome the closest NRPS in homology was AFLA_064540 (48 % aa identity and 67 % positive). AFLA_064540 is in a gene cluster 21 and has homology to numerous other genes predicted to encode proteins involved in biosynthesis of an epithiopiperazine metabolite similar to gliotoxin. Maximum homology to other NRPSs in fungi was ≤ 40% aa identity. The recognized conserved domains were an adenylation (A) domain (aa 24-505), a phosphopantetheinyl (pp) attachment site (aa 536-584), and a SDR/thioreductase domain (aa 643-938). In gramicidin B biosynthesis, one of its many protein binding domains recognizes Trp while in gramicidin C biosynthesis the recognized aa is Phe. We prepared a deletion mutant of the NRPS (AFLA_023020) gene in *A. flavus* CA14 by homologous recombination using pyrithiamine selection. Extracts of the wild type untransformed fungi showed a LC peak at 15.1 min that was not present in the NRPS knockout mutant (Fig. 3). This peak contained a metabolite with *m/z* 693.3182 (calculated for ditryptophenaline $C_{42}H_{41}N_6O_4$ $[M+H]^+$: *m/z* 693.3184). An ion corresponding to the monomer form was also apparent with *m/z* 346.1548. Comparison with an authentic standard of DTP gave identical elution and *m/z* values as well as the same MS^n profiles as for the standard. DTP had previously been identified as a metabolite from *A. flavus* and other related fungi (Varga, *et al.*, 2012).

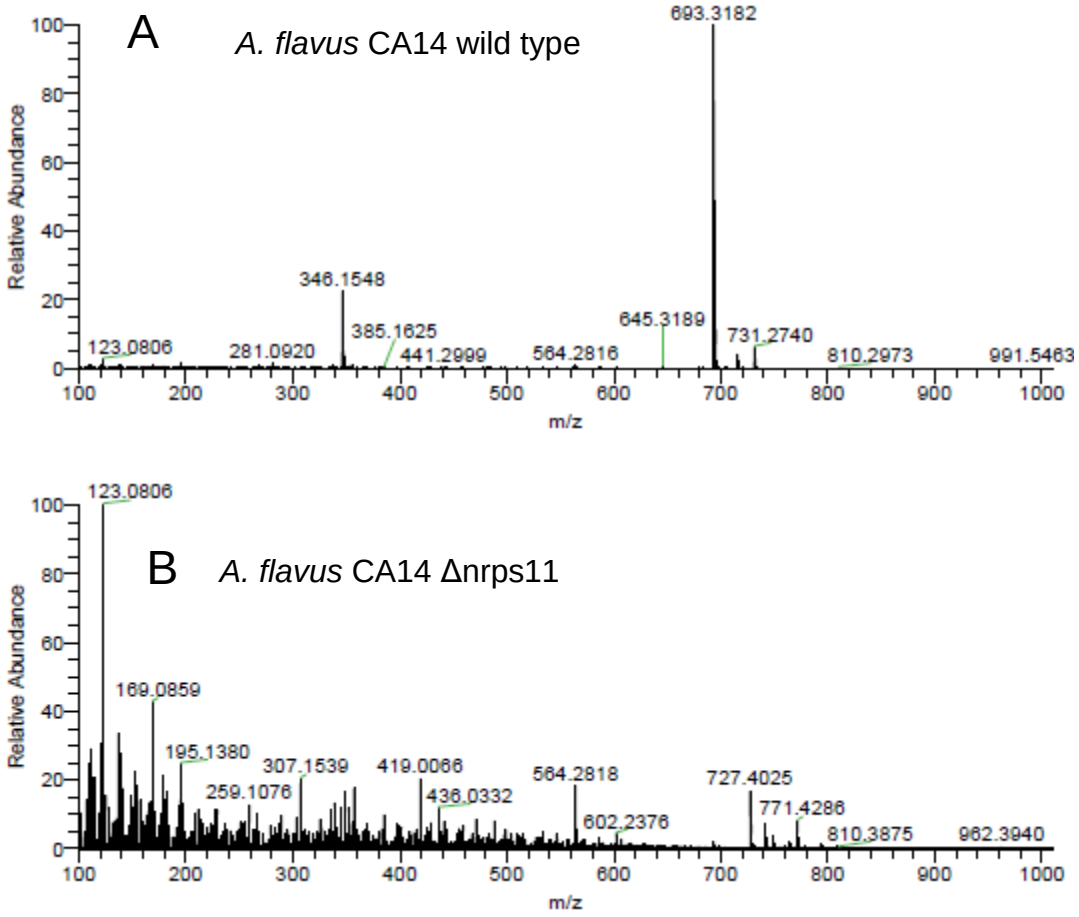

Fig.3. LC-MS peaks for ditryptophenaline in wild type (panel A) and AFLA_023020 (*nrps*) deletion mutant (panel B). ESI^+, full MS, mass range: *m/z* 100-1000

Several of the genes neighboring AFLA_023020 were also expressed under similar conditions of growth. These included genes encoding a possible methylase/desaturase (AFLA_023010), a GliC like cytochrome P450 (AFLA_023030), an integral membrane, ankyrin-repeat domain protein (AFLA_023000), a putative MFS transporter (AFLA_023050), and a gene with a ferredoxin reductase domain (Table 1). The CypX-type cytochrome P450 monooxygenase (AFLA_023030) resembled cytochrome P450 enzymes from gliotoxin biosynthesis in *Trichoderma virens* and *A. fumigatus* (GliC) with 50 % and 38 % aa identity (68 % and 59 % positive aa) respectively. Knockout of this gene gave a mutant capable of only producing DTP at in amounts about 12.5 % that of the wild type (Table 3; Fig. 4). Similarly knockout mutagenesis of the presumptive transcription factor (AFLA_023040) also gave a mutant with reduced ability to synthesize DTP.

Table 3 Comparison of signal intensities for ditryptophenaline in different cluster 11 mutants

Gene mutation	Putative function	*m/z* 693.3182
None	Wild type	225[a]
ΔAFLA_023010	GA4 desaturase	25
ΔAFLA_023020	NRPS	0
ΔAFLA_023030	Cytochrome P450	30
ΔAFLA_023040	Zn_2Cys_6 TF	52

[a]Signal abundance x 10^5

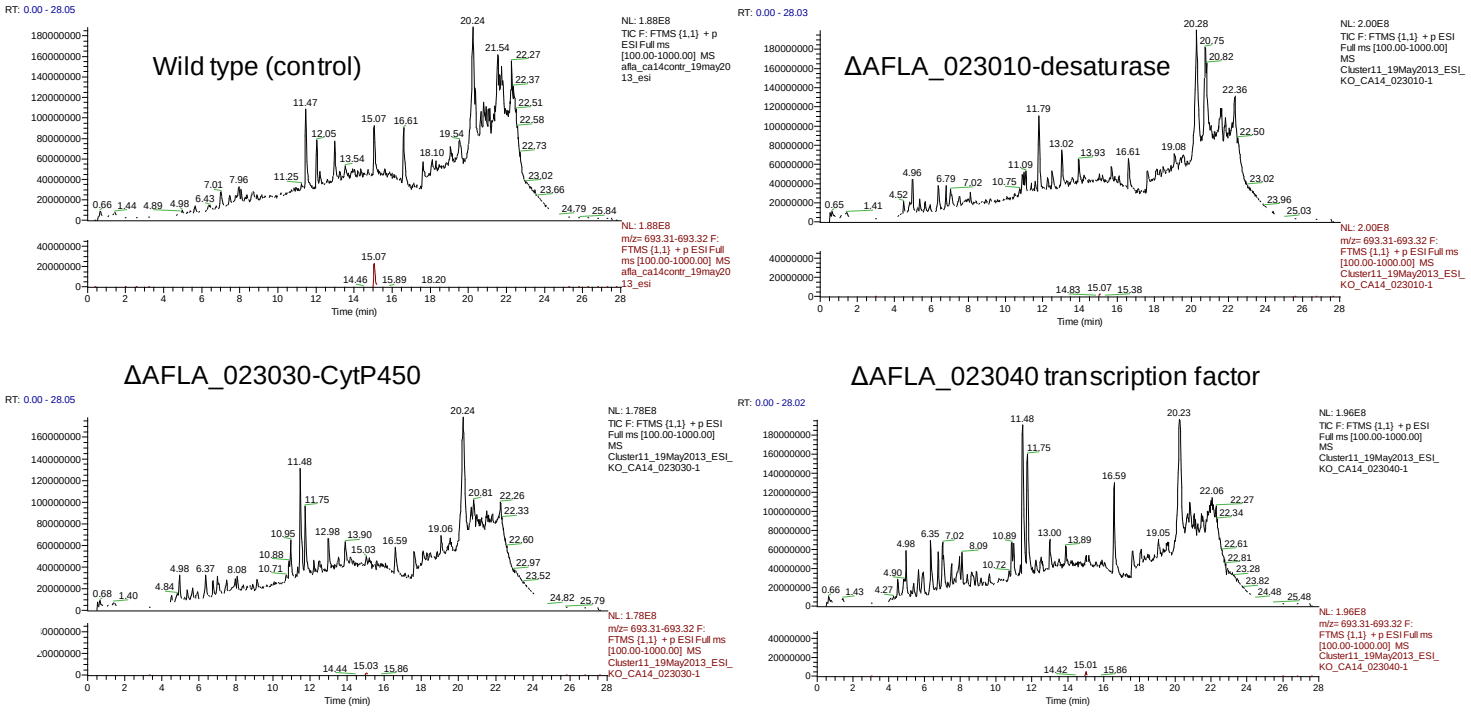

Fig. 4. Comparison of abundance of ditryptophenaline $[M+H]^+$ ion in wild type and mutants of putative ditryptophenaline biosynthesis genes.

Another gene in cluster 11 (AFLA_023010) is predicted to encode a 227 aa GA4 desaturase, although a desaturase domain was not specifically identified by Conserved Domain Search in NCBI. This gene has 63% homology to a 299 aa methyltransferase-like protein in *Beauveria bassiana* (GenBank accession number EJP68960). Based on this Blast analysis many similar proteins are found in fungi to which as yet no function has been

ascribed. We suggest that this enzyme catalyzes the N-methylation of the DTP precursor prior to oxidation and that the related enzymes may have a similar function in other fungi. Formation of DTP requires methylation of one of the N residues of the diketopiperazine ring and this step is probably necessary before oxidation can occur to allow the formation of the pyrrolidinoindoline ring. Insertional mutation of this gene gave a transformant still able to produce DTP but at approximately one-tenth the amount as estimated by LC-MS. Two other genes identified as encoding proteins in the cluster are AFLA_023070 and AFLA_023080. Both are characterized as integral membrane proteins. AFLA_023070, a predicted 523 aa protein possesses a RGS (regulator of G-protein signaling) domain while AFLA_023080 is similar to TmpA in *A. nidulans*, a membrane flavoprotein that was shown to be involved in production of a metabolite which regulates asexual development (Soid-Raggi, *et al.*, 2006). This protein was considered to be a membrane oxidoreductase.

We also examined extracts of the close relatives of *A. flavus* for the presence of DTP. These results showed that a variant *A. flavus* with small sclerotia (AF70), *A. parasiticus*, and *A. nomius* are able to produce DTP under the same conditions of growth as that of *A. flavus* (Fig. 5).

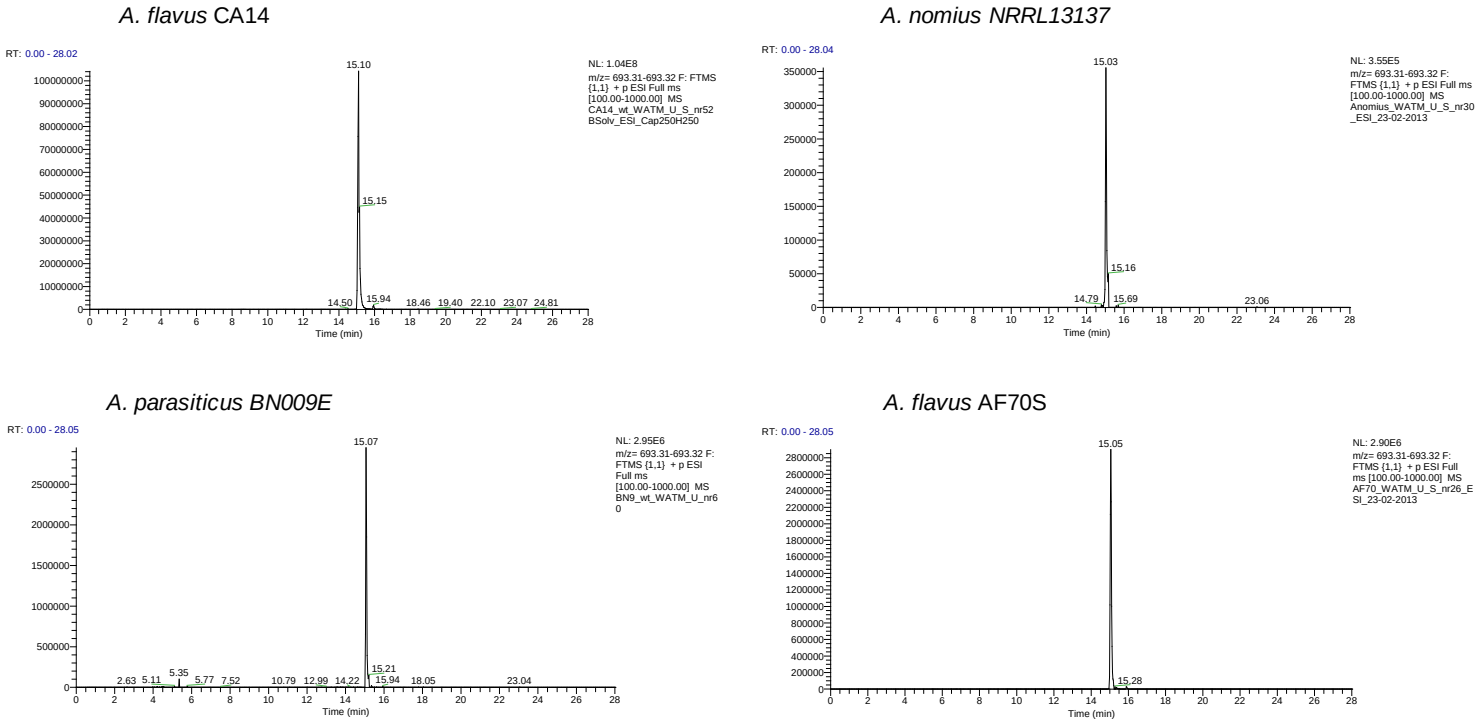

Fig. 5. Presence of ditryptophenaline in related aflatoxin-producing species.

Our study provides strong evidence that the cluster 11 NRPS catalyzes the production of the indole-diketopiperazine dipeptide that serves as the initial metabolite for the formation of DTP (Fig. 6). With the identification of several of the other modification genes in the cluster we propose a plausible biosynthesis scheme (Fig. 6). The order of the conversion steps is speculative with methylation shown as the first step after the NRPS catalysis, followed by subsequent oxidation steps. Our results suggest that although these genes are important for biosynthesis, the biosynthesis is "leaky" and other related genes may encode enzymes that could weakly catalyze these intermediate steps. Such "cross-talk" among gene clusters has been shown for the genes encoding the related piperazines produced by *A. flavus* clusters 35 and 48 (Forseth, *et al.*, 2013). Cluster 11 lacks the reductive steps involving NmrA-like proteins in the related clusters 35 and 48 in *A. flavus* that are necessary for reduction of the pyrazines intermediate to produce the piperazines, LnaA and LnaB (Forseth, *et al.*, 2013). Although we did not prepare knockout mutants of AFLA_023070 or AFLA_023080, the former gene is not present in the other fungi studied that still produce DTP and is unlikely to be involved in biosynthesis, but could be involved in some other function of DTP. The latter gene, a putative ferridoxin reductase, is likely to be necessary for the dimerization. Bio-inspired oxidations have been used for other tryptophan dimerizations (Tadano, *et al.*, 2013). We suggest that the ferridoxin reductase catalyzes the dimerization of the DTP monomer precursor.

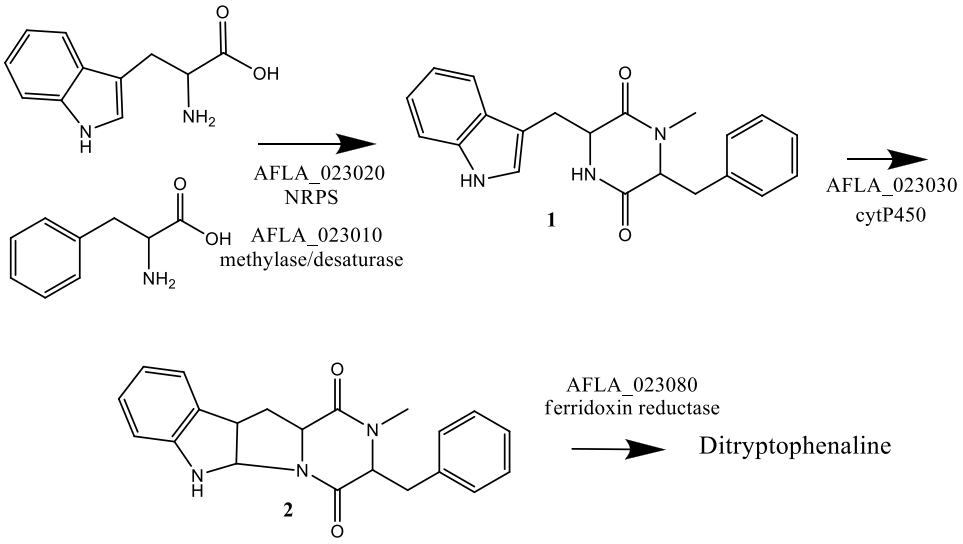

Fig. 6. Proposed scheme for biosynthesis of ditryptophenaline by enzymes encoded by genes in *A. flavus* cluster 11

DTP has received attention for its potential pharmaceutical use as a substance P antagonist (Popp, *et al.*, 1994). Substance P is an undecapeptide neurotransmitter and neuromodulator and belongs to the tachykinin neuropeptide family. Substance P is an important element in pain perception. Substance P receptor antagonists are important for potential treatment of stress-related illnesses and for remediation of pain. The function of DTP in the fungus has not been studied. The recently discovered piperadines from NRPSs LnaA and LnaB haved been linked to a signaling network involved in formation of sclerotia by *A. flavus* (Forseth, *et al.*, 2013). Although DTP is structurally different from the LnaA and LnaB metabolites, given its activity as a substance P antagonist (Popp, *et al.*, 1994), it is entirely possible that it could serve to mediate signal transmission in *A. flavus*.

CONCLUSIONS

Aspergillus flavus is known mainly for its ability to produce the toxic and carcinogenic aflatoxins; it also produces numerous other secondary metabolites, some of which are potentially beneficial. In the present project, gene cluster 23 overexpressed mutants and gene cluster 11 knockout mutants were made for *A. flavus*, and a general sample pretreatment method was established for extraction of various metabolites of fungi in different matrices. Then, the metabolite profiles of the knockout/overexpressing mutants was compared to those of the wild-type strains by the developed UHPLC-Orbitrap-HRMS and LC-Ion-Trap methods to determine the function of gene cluster 11 in *A. flavus.* The results showed that the known *A. flavus* metabolite, ditryptophenaline (DTP), is formed by genes in a cluster containing a 1021 aa non-ribosomal peptide synthase with a domain structure similar to those involved in formation of diketopiperazines and epidithiodioxopiperazines. Besides a gene for an AflR-like Zn_2Cys_6 transcription factor, two other genes in the cluster are predicted to encode a desaturase and a cytochrome P450 monooxygenase that are likely to be involved in DTP biosynthesis. Identification of this biosynthetic gene cluster should allow preparation of new members of this important class of Substance P neurotransmitter antagonists. Based on the observations, all the aims set in the present project have been achieved.

PUBLICATIONS

1. Manuscripts

(1) José Diana Di Mavungu, **Zheng Han**, Svetlana V. Malysheva, Brian M. Mack, Sarah De Saeger & Kenneth C. Ehrlich. Identification of the probable gene cluster in *Aspergillus flavus* for ditryptophenaline biosynthesis. Submitted to FEMS Letters.

(2) Investigation of the *Aspergillus flavus* cluster 23 metabolites by high resolution and multiple stage mass spectrometry. In preparation (Collaboration between Ghent University and Southern Regional Research Center, ARS/USDA, I will be one of the authors in this paper) .

(3) Determination of aflavarin and its derivatives to clarify a novel function of gene cluster 39 in *Aspergillus flavus*. In preparation (Collaboration between Ghent University and Southern Regional Research Center, ARS/USDA, I will be one of the authors in this paper).

2. Oral presentation

(1) **De Saeger S**. Challenges and applications in untargeted-based mycotoxin research. "International Mycotoxin Conterences 2014", May 19-23, 2014, Beijing, China.

(2) **José Diana Di Mavungu**. Using comparative metabolomics to determine secondary metabolite gene function in *Aspergillus flavus*. SINO-BELGIAN BILATERAL R&D – COOPERATION " Mycotoxins and mycotoxigenic fungi in China: analytical tools, dietary exposure and Fusarium diversity" (2012-2014). Second Workshop and Users meeting, May 21, 2013 –Ghent University, Ghent, Belgium

REFERENCES

Andersen, MR, Salazar, MP, Schaap, PJ, van deVondervoort, PJI, Culley, D, Thykaer, J, et al. (2011) Comparative genomics of citric-acid-producing Aspergillus niger ATCC 1015 versus enzyme-producing CBS 513.88. Genome Res 21, 885-897.

Barrow, CJ & Sedlock, DM (1994) 1'-(2-Phenyl-ethylene)-ditryptophenaline, a new dimeric diketopiperazine from *Aspergillus flavus*. Journal of Natural Products 57, 1239-1244.

Chang, P-K, Horn, BW & Dorner, JW (2009) Clustered genes involved in cyclopiazonic acid production are next to the aflatoxin biosynthesis gene cluster in *Aspergillus flavus*. Fungal Genet Biol 46, 176-182.

Chang, PK, Scharfenstein, LL, Ehrlich, KC, *et al.* (2012) Effects of *laeA* deletion on *Aspergillus flavus* conidial development and hydrophobicity may contribute to loss of aflatoxin production. Fungal Biol 116, 298-307.

Chang, PK, Ehrlich, KC, & Fujii, I (2009) Cyclopiazonic Acid Biosynthesis of *Aspergillus flavus* and *Aspergillus o*ryzae. Toxins 1, 74-99.

Chang, PK, Yabe, K & Yu, J (2004) The *Aspergillus parasiticus estA*-encoded esterase converts versiconal hemiacetal acetate to versiconal and versiconol acetate to versiconol in aflatoxin biosynthesis. Appl Environ Microbiol 70, 3593-3599.

Cornacchia, C, Cacciatore, I, Baldassarre, L, *et al.* (2012) 2,5-diketopiperazines as neuroprotective agents. Mini Rev Med Chem 12, 2-12.

Forseth, RR, Amaike, S, Schwenk, D, *et al.* (2013) Homologous NRPS-like gene clusters mediate redundant small-molecule biosynthesis in *Aspergillus flavus*. Angew Chem Int Ed Engl 52, 1590-1594.

Frisvad, JC & Thrane, U (1987) Standardized high-performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV-VIS spectra (diode array detection). J Chromatogr A 404, 195-214.

Georgianna, DR, Fedorova, ND, Burroughs, JL, *et al.* (2010) Beyond aflatoxin: four distinct expression patterns and functional roles associated with *Aspergillus flavus* secondary metabolism gene clusters. Mol Plant Pathol 11, 213-226.

Han, Z, Ren, YP, Zhu, J, Cai, Z, Chen, Y, Luan, L, et al. (2012) Multianalysis of 35 Mycotoxins in Traditional Chinese Medicines by Ultra-High- Performance Liquid Chromatography-Tandem Mass Spectrometry Coupled with Accelerated Solvent Extraction. J Agr Food Chem 60, 8233-8247.

Khaldi, N, Seifuddin, FT, G., T, *et al.* (2010) SMURF: genomic mapping of fungal secondary metabolite clusters. Fungal Genet Biol 47, 736-741.

Lu, DY & Lu, TR (2010) Antimetastatic activities and mechanisms of bisdioxopiperazine compounds. Anticancer Agents Med Chem 10, 564-570.

Malysheva, SV, Arroyo-Manzanares, N, Cary, JW, Ehrlich, KC, Vanden Bussche, J, Vanhaecke, L, Bhatnagar, D, Diana Di Mavungu, J, De Saeger, S. (2014) Identification of novel metabolites from *Aspergillus flavus* by high resolution and multiple stage mass spectrometry. *Food Additives and Contaminants: Part A*, DOI: 10.1080/19440049.2013.859743

Movassaghi, M, Schmidt, MA & Ashenhurst, JA (2008) Concise total synthesis of (+)-WIN 64821 and (-)-ditryptophenaline. Angew Chem Int Ed Engl 47, 1485-1487.

Nicholson, MJ, Koulman, A, Monahan, BJ, Pritchard, BL, Payne, GA, & Scott, B (2009) Identification of two aflatrem biosynthesis gene loci in Aspergillus flavus and metabolic engineering of Penicillium paxilli to elucidate their function. Appl Environ Microbiol 75(23), 7469-7481.

Overman, LE & Paone, DV (2001) Enantioselective total syntheses of ditryptophenaline and ent-WIN 64821. J Am Chem Soc 123, 9465-9467.

Popp, JL, Musza, LL, Barrow, CJ, *et al.* (1994) WIN 64821, a novel neurokinin antagonist produced by an *Aspergillus* sp. III. Biosynthetic analogs. J Antibiot (Tokyo) 47, 411-419.

Reddy, KRN, Salleh, B, Saad, B, *et al.* (2010) An overview of mycotoxin contamination in foods and its implications for human health. Toxin Reviews 29, 3-26.

Ren, YP, Zhang, Y, Shao, S, Cai, Z, Feng, L, Pan, H et al. (2007) Simultaneous determination of multi-component mycotoxin contaminants in foods and feeds by ultra-high-performance liquid chromatography tandem mass spectrometry. J Chromatogr A 1143, 48-64.

Scharf, DH, Chankhamjon, P, Scherlach, K, *et al.* (2013) Epidithiodiketopiperazine biosynthesis: a four-enzyme cascade converts glutathione conjugates into transannular disulfide bridges. Angew Chem Int Ed Engl 52, 11092-11095.

Smedsgaard, J (1997) Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. J Chromatogr A 760, 264-270.

Soid-Raggi, G, Sanchez, O & Aguirre, J (2006) TmpA, a member of a novel family of putative membrane flavoproteins, regulates asexual development in *Aspergillus nidulans*. Mol Microbiol 59, 854-869.

Springer, JP, Kobbe, B, Demain, AL, *et al.* (1977) The structure of ditryptophenaline-a new metabolite of *Aspergillus flavus*. Tetrahedron Lett 2403-2406.

Sulyok, M, Berthiller, F, Krska, R & Schuhmacher, R (2006) Development and validation of a liquid chromatography-tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. Rapid Commun Mass Spectrom 20, 2649–2659.

Sulyok, M, Krska, R & Schuhmacher, R (2007) 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 389,1505–1523.

Szewczyk, E, Chiang, YM, Oakley, CE, *et al.* (2008) Identification and Characterization of the Asperthecin Gene Cluster of *Aspergillus nidulans*. Appl Environment Microbiol 74, 7607-7612.

Tadano, S, Mukaeda, Y & Ishikawa, H (2013) Bio-inspired dimerization reaction of tryptophan derivatives in aqueous acidic media: three-step syntheses of (+)-WIN 64821, (-)-ditryptophenaline, and (+)-naseseazine B. Angew Chem Int Ed Engl 52, 7990-7994.

Varga, J, Frisvad, JC & Samson, RA (2012) Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. Stud Mycol 69, 57-80.

Yu, J, Bhatnagar, D & Cleveland, TE (2004) Completed sequence of aflatoxin pathway gene cluster in Aspergillus parasiticus. FEBS Letters 564(1-2), 126-130.