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Ochratoxin A production by *Penicillium thymicola*

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin produced by some *Aspergillus* and *Penicillium* species that grow on economically important agricultural crops and food products. OTA is classified as Group 2B carcinogen and is potently nephrotoxic, which is the basis for its regulation in some jurisdictions. Using high resolution mass spectroscopy, OTA and ochratoxin B (OTB) were detected in liquid culture extracts of *Penicillium thymicola* DAOMC 180753 isolated from Canadian cheddar cheese. The genome of this strain was sequenced, assembled and annotated to probe for putative genes involved in OTA biosynthesis. Known OTA biosynthetic genes from *Penicillium verrucosum* or *Penicillium nordicum*, two related *Penicillium* species that produce OTA, were not found in *P. thymicola*. However, a gene cluster containing a polyketide synthase (PKS) and PKS-nonribosomal peptide synthase (NRPS) hybrid encoding genes were located in the *P. thymicola* genome that showed a high degree of similarity to OTA biosynthetic enzymes of *Aspergillus carbonarius* and *Aspergillus ochraceus*. This is the first report of ochratoxin from *P. thymicola* and a new record of the species in Canada.

Introduction

The mycotoxin ochratoxin A (OTA) is a natural agricultural contaminant produced by several *Penicillium* and *Aspergillus* species (Varga *et al.* 2001). OTA is potently nephrotoxic in rodents, pigs and poultry and exposure results in renal tumours in a variety of animal species (Barlow *et al.* 2008; Bondy *et al.* 2015). OTA is classified as a possible human carcinogen (Group 2B) by the International Agency of Research on Cancer (IARC 1993). The European Commission has several regulations on OTA in various commodities (van Egmond *et al.* 2007). These regulations directly affect the trade of raw materials and finished food products globally. This can affect the entire food production chain all the way to farmers and food producers (Miller *et al.* 2014).

Two sister species of *Penicillium* sect. *Fasiculata* (Frisvad & Samson 2004) are important OTA producers in food; *Penicillium verrucosum* on grains (Larsen *et al.* 2001a; Lund & Frisvad 2003)

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and *Penicillium nordicum* on dried, salted meats (Cabañes *et al.* 2010; Sonjak *et al.* 2011). The growth of *P. verrucosum* on damp grains in storage is a major source of OTA and results in significant human exposure, either by direct consumption of contaminated products or indirectly via products made from animals fed contaminated feed (Duarte *et al.* 2010). *Penicillium nordicum* produces more OTA than *P. verrucosum* and is a serious concern in some European countries, where cured meats are consumed regularly. *Penicillium thymicola* is a recently described species, isolated from dried thyme, but also from soil and sorghum grain (Frisvad & Samson 2004). Not previously known to produce OTA, although European strains synthesize the amino acid derived compounds alantrypinone and fumiquinazoline F, and the alpha-pyrone PC-2 (Larsen *et al.* 1998; Frisvad & Samson 2004).

Thirteen species of *Aspergillus* produce OTA, mainly in sections *Circumdati* (Visagie *et al.* 2014a) and *Nigri* (Samson *et al.* 2004). Although the occurrence of OTA in beer is a consequence of *P. verrucosum* growing on barley, the occurrence of OTA in grapes, wine, cocoa and coffee is probably attributable to contaminating *Aspergillus* species (Barlow *et al.* 2008; Kuiper-Goodman *et al.* 2010; Nguyen & Ryu 2014; Lee & Ryu 2015), such as *Aspergillus carbonarius* or some strains of *Aspergillus niger* (Varga *et al.* 2011).

The chemical structure of OTA consists of a polyketide-derived dihydroisocoumarin moiety linked via the carboxyl group of the amino acid phenylalanine (Harris & Mantle 2001). Despite the negative health effects caused by OTA, little is currently known about the genetics of its biosynthesis. It is possible that a polyketide synthase (PKS) catalyses the formation of a dihydroisocoumarin pentaketide intermediate that is subsequently chlorinated by a chloroperoxidase or halogenase and the amino acid phenylalanine is then linked to the polyketide intermediate by a non-ribosomal peptide synthase (NRPS) (Harris & Mantle 2001; el Khoury & Atoui 2010). Fungal PKS's consist of multiple catalytic domains with ketosynthase (KS), acetyltransferase (AT), and acyl carrier protein (ACP) domains. Additional processing domains, including ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), cyclase, thioesterase and methyltransferase functions, are also often present within a single PKS complex (Keller *et al.* 2005). A typical NRPS consists of adenylation (A), thiolation (T), condensation (C), and terminal release domains (Schwarzer *et al.* 2003). Functional characterizations of some PKS and NRPS genes have been performed by gene inactivation and expression experiments in *P. nordicum* (Karolewiez & Geisen 2005; Geisen *et al.* 2006), *P. verrucosum* (Schmidt-Heydt *et al.* 2007; Abbas *et al.* 2013), *A. carbonarius* (Gallo *et al.* 2009; Gallo *et al.* 2012; Gallo *et al.* 2014), *Aspergillus ochraceus* (O'Callaghan *et al.* 2003), and *Aspergillus westerdijkiae* (Bacha *et al.* 2009).

During a large study screening Canadian isolates of *P. verrucosum* and related species for OTA, OTA, and OTB were unexpectedly identified by high-resolution mass spectrometry (HRMS) from a culture filtrate extract of *P. thymicola* DAOMC 180753, originally isolated from cheddar cheese. Because of the close evolutionary relationship of *P. thymicola* to *P. nordicum*, and *P. verrucosum*, their metabolite profiles were further compared. A genomic analysis of *P. thymicola* was undertaken to locate putative genes required for OTA biosynthesis. Here, we report the production of OTA and OTB, locate putative OTA biosynthetic genes using genomic data and document the occurrence of *P. thymicola* in Canada.

Materials and methods

Chemical detection of OTA

An agar slant of *Penicillium thymicola* DAOMC 180753 growing on 2 % Blakeslee's malt extract agar (Visagie *et al.* 2014b) was macerated in sterile distilled deionized water and a 5 % (v/v) aliquot was used to inoculate nine 250 mL Erlenmeyer flasks containing 100 mL of yeast extract sucrose media (30 g L^{-1} yeast extract (Sigma, USA) and 150 g L^{-1} sucrose (BioShop, Canada)). Flasks were incubated on a rotary shaker (100 rpm at 25 °C) for 5 d.

After the incubation period, culture filtrates of three individual flasks were combined, resulting in three replicates for subsequent analysis. From these, the mycelia were separated from the filtrate by vacuum filtration through Whatman #4 filter paper (Whatman GE Healthcare, UK) and the resulting volume of media recorded. Mycelia were subsequently frozen at −20 °C, lyophilized and weighed. Each culture filtrate was saturated with NaCl and extracted with three equal volumes of ethyl acetate. The organic layer was filtered through a Whatman #1 (Whatman GE Healthcare, UK) and anhydrous sodium sulfate prior to drying by rotary evaporation. The crude extract was dissolved in a minimal amount of HPLC grade methanol, filtered through 13 mm PTFE, 0.2 μm syringe filter (Tisch Scientific, USA) and dried under a gentle stream of nitrogen gas. Extracts were stored dry in amber vials at −20 °C. Additionally, extracts of *Penicillium nordicum* DAOMC 158653 and *Penicillium verrucosum* DAOMC 242724 were prepared as reported above.

Crude filtrate extracts were dissolved in 300 μL of HPLC grade methanol and analysed for OTA by liquid chromatography tandem mass spectrometry (LC-MSMS) in multiple reaction monitoring (MRM) mode. This was achieved using a Waters 2795 separations module and MicroMass Quatro Ultima triple quadrupole mass spectrometer (Waters Corporation, USA). Extracts were separated with a Kinetix C18 (100 × 4.60 mm, 2.6 μm) column (Phenomenex, USA) and a mobile phase consisting of acetonitrile-deionized water with 0.1 % formic acid (v/v). The solvent gradient was a linear program that increased from 5 to 100 % acetonitrile over 13 min with a flow rate of 1.0 mL min^{-1}. Positive ESI conditions included: capillary voltage 3.50 kV, cone voltage 20 V, source temperature 80 °C, desolvation temperature 180 °C, cone gas flow (N2) 90 L h^{-1}, desolvation gas flow (N2) 540 L h^{-1}, collision gas (Ar) pressure 2.3×10^{-4} mbar and multiplier voltage 650 V. LC-MSMS analysis of OTA used two transitions, *m/z* 404 → 358 and *m/z* 404 → 341, with the same collision energy, 20 eV. The *m/z* 358 transition was the quantifier whereas *m/z* 341 was the qualifier. A standard curve was constructed with an authentic OTA standard (Sigma, USA) ranging from 1 to 10 000 ng on column with an R2 value of 0.99 over the entire concentration range.

HRMS and tandem high resolution mass spectrometry (HRMS2) of *Penicillium* extracts were acquired with a Q-Exactive Quadrupole Orbitrap Mass Spectrometer (Thermo Scientific, USA), coupled to a 1290 HPLC system (Agilent, USA) in

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ESI positive mode (scan range 100–1500 *m/z*). Metabolites were separated with a Zorbax Eclipse Plus RRHD C18 (2.1 × 50 mm, 1.8 μm) column (Agilent, USA) maintained at 35 °C and a mobile phase consisting of acetonitrile-deionized water with 0.1 % formic acid (v/v). Acetonitrile was held at 0 % for 0.5 min, before increasing to 100 % over 3.5 min and held at 100 % for 1.5 min before returning to 0 % over 0.5 min at a flow rate of 0.3 mL min^{-1}. Authentic standards of OTA (Sigma, USA), citrinin (Sigma, USA), and phenylalanine (Sigma, USA) were utilized in conjunction with high resolution mass data to identify secondary metabolites.

Growth, DNA extraction and genome sequencing

Penicillium thymicola DAOMC 180753 was grown on Blakeslee's malt extract agar for 7 d at 25 °C (Visagie *et al.* 2014b). Colonies were flooded with 5 mL of sterile distilled water to make a spore suspension. One mL was inoculated in 100 mL of Blakeslee's malt extract broth and was left on a rotary at 300 rpm at 25 °C for 6 d. To isolate fungal culture pellets for DNA extraction, the broth was removed by filtration. DNA was extracted with the OmniPrep kit for fungi (G-Biosciences, St. Louis, Missouri, USA) following manufacturer's instructions. DNA extracts were combined and whole-genome sequencing (paired-end with 101 base pairs (bp)) was performed on an Illumina HiSeq 2500 with TrueSeq V3 chemistry at the National Research Council Canada in Saskatoon, Saskatchewan, Canada.

Genome assembly and annotation

Quality of the reads was checked with the program FastQC v0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Using fastx_trimmer (part of the FASTX-Toolkit v0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/)), 10 bases from the 5′ end were trimmed to yield 91 bp reads of higher quality. Afterwards, adaptor sequences were removed with Trimmomatic v0.33 (Bolger *et al.* 2014). The optimal k parameter was predicted with KmerGenie v1.6950 (Chikhi & Medvedev 2014). *De novo* genome assembly was performed with SPAdes v3.5.0 (Bankevich *et al.* 2012) and error correction using BayesHammer (Nikolenko *et al.* 2013) with mismatch correction enabled. Scaffolds shorter than 1000 bp were discarded. Assembly statistics were generated with QUAST v2.3 (Gurevich *et al.* 2013). The assembly was checked by alignment of the corrected reads onto the scaffolds using Bowtie2 v2.0.0 (Langmead & Salzberg 2012). Alignments produced by Bowtie2 in SAM format were converted to sorted BAM format by SAMtools v0.1.19 (Li *et al.* 2009) and statistics for coverage were generated with Qualimap v2.1 (Garcia-Alcalde *et al.* 2012). To assess the completeness of our genome assembly, CEGMA v2.5 (Parra *et al.* 2007) was run on the scaffolds to detect the percentage of conserved eukaryotic genes (CEG's).

Gene annotation was performed using the JGI Annotation pipeline (Grigoriev *et al.* 2006). Subsequently, the antibiotics & Secondary Metabolite Analysis Shell (antiSMASH) v2.1.1 (Blin *et al.* 2013) was used to predict gene clusters involved in secondary metabolite production with annotations generated from the JGI annotation pipeline. All data are available for download on the JGI MycoCosm portal (Grigoriev *et al.* 2014) (jgi.doe.gov/fungi) and raw reads are available on NCBI SRA (ncbi.nlm.nih.gov/sra) under accession number SRR1909079.

Results

Secondary metabolite profiles of Penicillium thymicola, Penicillium verrucosum, *and* Penicillium nordicum

The secondary metabolite profiles of *Penicillium thymicola* DAOMC 180753, *Penicillium verrucosum* DAOMC 242724, and *Penicillium nordicum* DAOMC 158653 were investigated by HRMS approaches and these data are presented in Table 1. Each of the three *Penicillium* sister species produced OTA as determined by comparisons of high resolution product ion spectra to the literature (Nielsen & Smedsgaard 2003; Sulyok *et al.* 2006; Varga *et al.* 2013) and an authentic standard. HRMS analysis of the *P. thymicola* extract revealed an $[M+H]^+$ OTA precursor ion at *m/z* 404.08945 (1.6 ppm mass error) and product ions at *m/z* 358.08438, 341.05795, 257.02130, and 239.01070 (Fig 1). The retention index and mass spectra of OTA from the crude extract were compared to the authentic standard and found to be identical, unambiguously identifying the mycotoxin OTA within the culture filtrate extract of *P. thymicola*. Each *Penicillium* species culture filtrate extract was also analysed by LC-MRM mass

Table 1 – Secondary metabolite production by strains of *P. thymicola*, *P. nordicum* and *P. verrucosum*. OTA, citrinin and phenylalanine were verified by an authentic standard (marked by an asterisk). The + indicates the compound was detected while the – indicates the compound was not detected. It should be noted that phenylalanine is a primary metabolite important for OTA biosynthesis and not a secondary metabolite.

Secondary metabolite	*P. thymicola* DAOMC 180753	*P. nordicum* DAOMC 158653	*P. verrucosum* DAOMC 242724
OTA*	+	+	+
OTB	+	+	+
Citrinin*	–	–	+
Alantrypinone	+	–	–
Fumiquinazoline F	+	–	–
Phenylalanine*	+	+	+
PC-2	+	+	+
Sclerotigenin	–	+	–
Lumpidin	–	–	–

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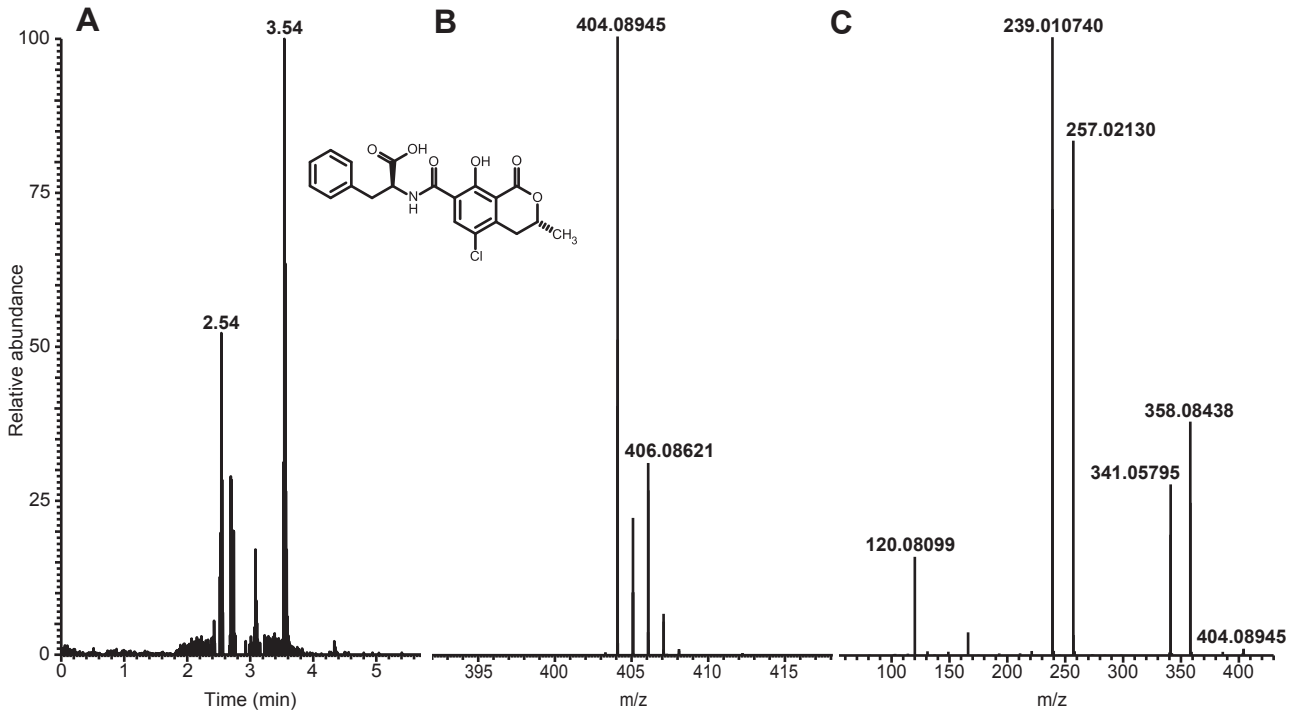

Fig 1 – Extracted ion chromatogram for OTA (A; 3.54 min; chemical structure illustrated), HRMS spectra in positive mode where the chlorine isotope profile is visualized (B) and HRMS^2 fragmentation ions for OTA (C) produced by *P. thymicola* DAOMC 180753.

spectrometry. The OTA precursor ion at *m/z* 404.0 $[M+H]^+$ was selected and quantitation was achieved using the *m/z* 358.0 product ion, which is in agreement with the results of Nielsen & Smedsgaard (2003). Using the reported LC-MRM conditions, *P. thymicola* DAOMC 180753 produced 7.5 ± 1.5 ng OTA/mL/g dry cells, *P. verrucosum* DAOMC 242724 39.1 ± 1.5 ng OTA/mL/g dry cells and *P. nordicum* DAOMC 158653 276.3 ± 43.1 ng OTA/mL/g dry cells. OTB, the dechlorinated form of OTA, and phenylalanine were also synthesized by each *Penicillium* species (Table 1). From the *P. thymicola* extract, an OTB precursor $[M+H]^+$ ion at *m/z* 370.12860 (1.3 ppm mass error) and product ions at *m/z* 307.09845, 223.06009, and 205.04970 were observed, characteristic of this metabolite (Nielsen & Smedsgaard 2003). Fumiquinazoline F (*m/z* 359.15024 $[M+H]^+$; 1.6 ppm mass error) and alantrypinone (*m/z* 373.12967 $[M+H]^+$; 1.1 ppm mass error; $C_{21}H_{16}N_4O_3$) were produced as major metabolites by this strain. The alpha-pyrone PC-2 was identified based on high-resolution mass (*m/z* 213.11217 $[M+H]^+$; 0.23 ppm mass error; $C_{11}H_{17}O_4$) from *P. thymicola*. At the same retention index, this metabolite was also detected in the two other *Penicillium* species extracts. The polyketide citrinin was only detected from *P. verrucosum*. *Penicillium nordicum* is suspected to produce sclerotigenin based on a $[M+H]^+$ ion at *m/z* 278.09286 (0.36 ppm mass error; $C_{16}H_{11}N_3O_2$) and appropriate retention index. A product ion of this metabolite at *m/z* 249.06618 and a large proportion of the precursor ion were observed in its HRMS^2 spectra. Neither lumpidin nor verrucines A and B were detected in any of the culture filtrate extracts examined based on interpretation of HRMS data.

Genome sequencing and characterization

The genome of *Penicillium thymicola* DAOMC 180753 was sequenced using a whole genome shotgun-sequencing strategy to locate putative OTA biosynthetic gene cluster(s). Approximately 28 million reads were assembled into 2167 scaffolds. The genome size was 33.9 Mb with a GC content of 47.9 %. The N50 value was 45.4 kb and the longest scaffold was approximately 215 kb. The median nucleotide coverage across the whole assembly was 53.7×. The assembled genome had a CEGMA score of 94.8 % calculated from the complete gene set and 97.2 % when calculated from both partial and complete gene sets. A total of 12 467 protein-coding genes were predicted. Of all predicted gene models, 11 240 were complete (90.2 %), but 1227 gene models lacked a start codon, stop codon or both (9.8 %). The mean gene length was 1582 bp, mean exon length was 462 bp and mean intron length was 90 bp.

Identification of secondary metabolite gene clusters

After the genome of *Penicillium thymicola* DAOMC 180753 was annotated, the bioinformatics program antiSMASH predicted a total of 99 secondary metabolite gene clusters, including 23 PKS, 17 NRPS, 6 PKS–NRPS hybrid, 13 terpene, 1 siderophore, and 39 labelled as putative or other.

To identify genes involved in OTA biosynthesis from *P. thymicola*, protein sequences from published OTA biosynthetic gene knockout and expression studies of *Penicillium* and *Aspergillus* species were queried. These sequences were compared

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Table 2 – Accession numbers of reference proteins involved in OTA biosynthesis and its putative homologue in *P. thymicola* DAOMC 180753.

Reference protein accession	Species	Study	*P. thymicola* protein accession	*P. thymicola* gene accession	% ID	E-value	Reference protein length	*P. thymicola* protein length	Length of alignment	antiSMASH cluster
gi|81176227|gb|AAT65816.2	*Penicillium nordicum*	Karolewiez & Geisen (2005)	jgi|Penth1|176637	gene_11104	96.98	0	398	399	398	Not detected
gi|30525891|gb|AAP32477.1	*Aspergillus ochraceus*	O'Callaghan et al. (2003)	jgi|Penth1|212294	gene_4875	71.38	3.00E-141	502	849	290	Cluster 39
jgi|Aspca3|173482|	*Aspergillus carbonarius*	Gallo et al. (2014)	jgi|Penth1|212294	gene_4875	70.59	5.00E-173	2541	849	374	Cluster 39
gi|193811752|emb|CAQ16344.1	*Aspergillus carbonarius*	Gallo et al. (2009)	jgi|Penth1|168734	gene_3514	70.4	0	729	3917	733	Not detected

to the predicted proteins from *P. thymicola* DAOMC 180753 with the BLASTp algorithm (Suppl. Table 1). Proteins with percent identity >70 % and an e-value < $1e^{-100}$ were considered strong matches. These were additionally verified to be arranged in secondary metabolite gene clusters. Protein sequences of three genes (*gene_4875*, *gene_11104*, and *gene_3514*) showed strong homology to proteins identified as involved in OTA biosynthesis in *Penicillium* (Karolewiez & Geisen 2005) and *Aspergillus* species (O'Callaghan *et al.* 2003; Bacha *et al.* 2009; Gallo *et al.* 2009; Gallo *et al.* 2012; Gallo *et al.* 2014) (Table 2).

Two of these genes making proteins homologous to known OTA biosynthetic enzymes were not organized within recognized gene clusters, but were located near the middle of genomic scaffolds. The *gene_11104 was* located on scaffold 785 (9430 bp long, positions 5059–6399). Its protein (jgi|Penth1|176637) showed homology to an alkaline serine protease from *Penicillium nordicum* (*aspPN*). The gene_3514 was located on scaffold 64 (88 623 bp long, positions 46 890–58 790) and encoded a 3916 amino acid protein (jgi|Penth1|168734) similar to the PKS enzyme from gene *ACpks* (GenBank CAQ16344.1) from *Aspergillus carbonarius* (Gallo *et al.* 2009). This *P. thymicola* protein was identified as producing a hybrid PKS–NRPS and had the following domains at various amino acid positions: KS from 44 to 467, AT from 575 to 890, DH from 967 to 1144, ER from 1649 to 1966, KR from 1992 to 2167, a C domain from 2388 to 2689, an A domain from 2881 to 3291, and a PCP domain from 3422 to 3489.

The *gene_4875* belongs to a type I PKS gene cluster called cluster 39 (Fig 2). Cluster 39 comprises 25 genes spanning 68.4 kb on scaffold 112 (Table 3). Within cluster 39, *gene_4875* encodes a 848 amino acid protein (jgi|Penth1|212294), that has an AT domain from position 280–581, *gene_4876* encodes a 332 amino acid protein (jgi|Penth1|170133) with an ER domain from position 6–260 and *gene_4877* encodes a 166 amino acid protein (jgi|Penth1|78053) with an ACP domain from position 104–151. The translated proteins of remaining genes within cluster 39 had poor matches to other proteins in the manually curated UniProt/Swiss-Prot protein database (Table 3).

Additionally, within the *P. thymicola* genome, there is a single halogenase (jgi|Penth1|178787) and two chloroperoxidase (jgi|Penth1|237379 and 238064) that could potentially catalyse the chlorination step in OTA biosynthesis.

Discussion

Here, we report the production of OTA and OTB from a Canadian strain of *Penicillium thymicola* isolated from cheddar cheese. Because of the close relationship of *P. thymicola* to well-documented ochratoxin-producing species of *Penicillium* (*Penicillium nordicum* and *Penicillium verrucosum*), a tight clade within *Penicillium* sect. *Fasciculata* (Frisvad & Samson 2004), their chemical profiles were compared (Table 1). Each of the three species produced OTA and its dechlorinated form, OTB, based on comparisons of experimental HRMS and $HRMS^2$ spectra to the literature and an authentic standard for OTA (Nielsen & Smedsgaard 2003; Sulyok *et al.* 2006; Varga *et al.* 2013). *Penicillium nordicum* produced the most

OTA of the strains studied, whereas *P. verrucosum* produced slightly more OTA compared to *P. thymicola*.

To date, OTA biosynthetic studies have only examined the inactivation of one gene and/or measure the expression of other genes encoded within an OTA gene cluster to investigate a correlation with its production. No study has been conducted where each gene in an identified OTA gene cluster was knocked out. It is not yet clear which genes, or protein domains, found within an OTA biosynthetic gene cluster are essential for its production.

The genome sequencing of *P. thymicola* DAOMC 180753 was originally undertaken as a negative control for comparative genomics of OTA gene clusters in *P. verrucosum* and *P. nordicum*. However, when OTA and OTB production were unexpectedly detected by HRMS, the genome was probed for putative OTA biosynthetic genes using protein sequences from known OTA genes of *Penicillium* and *Aspergillus* species. Secondary metabolite biosynthetic genes are often organized in clusters that encode key biosynthetic enzymes such as polyketide synthases (PKS's), non-ribosomal peptide synthases (NRPS's) and PKS–NRPS hybrid complexes, as well as both pathway-specific and global regulatory elements (Keller *et al.* 2005; Yu & Keller 2005). Initially, we thought that *P. thymicola* would have an OTA gene cluster similar to that of *P. nordicum* (Karolewiez & Geisen 2005; Geisen *et al.* 2006) or *P. verrucosum* (Schmidt-Heydt *et al.* 2007; Abbas *et al.* 2013). However, no proteins were identified from *P. thymicola* that were homologous with *P. verrucosum* (GenBank ABH05672.1) biosynthetic enzymes. One *P. thymicola* protein (jgi|Penth1|176637 from *gene_11104*) was homologous to an alkaline serine protease (*aspPN*) (GenBank AAT65816), previously found 6 kb distant from the *otapksPN* gene in *P. nordicum*, perhaps part of the

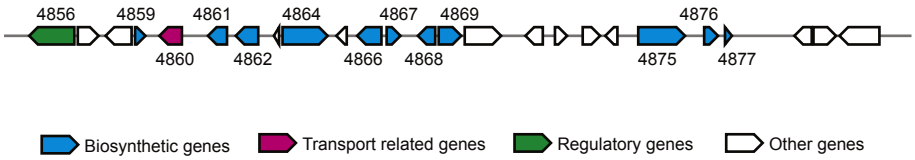

Fig 2 – Type I PKS gene cluster in *P. thymicola* DAOMC 180753 putatively involved in OTA biosynthesis.

Table 3 – Gene accession numbers, protein accession numbers and top UnitProt/SwissProt hit for genes in cluster 39.

P. thymicola gene accession	*P. thymicola* protein accession	antiSMASH gene type	UnitProt/SwissProt top hit (Aug 25, 2015)	% ID	E-value	Accession of hit
gene_4856	jgi|Penth1|153991	Regulatory gene	Ankyrin-2	28	2.00E-14	Q8C8R3.2
gene_4857	jgi|Penth1|249954	Other	Serine/threonine-protein kinase Nek5	26	0.024	Q7TSC3.1
gene_4858	jgi|Penth1|153997	Other	Vanillyl-alcohol oxidase	46	5.00E-169	P56216.1
gene_4859	jgi|Penth1|2433	Biosynthetic gene	15-hydroxyprostaglandin dehydrogenase [NAD(+)]	31	4.00E-29	P15428.1
gene_4860	jgi|Penth1|108108	Transport related gene	Uncharacterized MFS-type transporter	26	4.00E-41	O94343.1
gene_4861	jgi|Penth1|170113	Biosynthetic gene	Glutarate-semialdehyde dehydrogenase	52	5.00E-173	Q9I6M5.1
gene_4862	jgi|Penth1|108125	Biosynthetic gene	Pisatin demethylase	39	6.00E-111	Q12645.1
gene_4863	jgi|Penth1|212277	Other	4-carboxymuconolactone decarboxylase	42	5.00E-29	P20370.2
gene_4864	jgi|Penth1|108137	Biosynthetic gene	3-hydroxyisobutyrate dehydrogenase	36	2.00E-46	P29266.3
gene_4865	jgi|Penth1|108097	Other	No hits			
gene_4866	jgi|Penth1|108116	Biosynthetic gene	Lipase	29	5.00E-36	Q96VC9.1
gene_4867	jgi|Penth1|108123	Biosynthetic gene	Tropinone reductase-like 2	30	6.00E-21	H9BFQ1.1
gene_4868	jgi|Penth1|182132	Biosynthetic gene	Para-nitrophenol 4-monooxygenase	35	4.00E-72	C1I201.1
gene_4869	jgi|Penth1|108077	Biosynthetic gene	Fumitremorgin C synthase	28	1.00E-40	Q4WAW8.1
gene_4870	jgi|Penth1|212279	Other	Transcriptional regulator ADR1	47	3.00E-12	Q5AF56.1
gene_4871	jgi|Penth1|108111	Other	Sterol uptake control protein 2	55	1.00E-07	Q59QC7.1
gene_4872	jgi|Penth1|108098	Other	Protein RTM1	35	7.00E-42	P40113.1
gene_4873	jgi|Penth1|182137	Other	Zinc finger protein	29	2.1	P17017.3
gene_4874	jgi|Penth1|212286	Other	Peroxisomal catalase	44	3.3	Q9XZD5.1
gene_4875	jgi|Penth1|212294	Biosynthetic gene	Lovastatin nonaketide synthase	34	1.00E-64	Q0C8M3.2
gene_4876	jgi|Penth1|170133	Biosynthetic gene	Phthiocerol synthesis polyketide synthase type I	28	5.00E-26	P96202.2
gene_4877	jgi|Penth1|78053	Biosynthetic gene	Lovastatin diketide synthase	25	2.00E-05	Q9Y7D5.1
gene_4878	jgi|Penth1|249956	Other	Lipopolysaccharide-specific response protein	25	2.00E-09	Q8IXQ4.1
gene_4879	jgi|Penth1|249957	Other	RAS guanyl-releasing protein 4	28	3.3	Q1LZ97.1
gene_4880	jgi|Penth1|212313	Other	AMP deaminase	53	0	P50998.3

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same OTA gene cluster. However, *aspPN* was not inactivated by Karolewiez & Geisen (2005), leaving its impact on OTA biosynthesis ambiguous.

Considering that *P. thymicola* protein sequences did not have meaningful homologies with any *P. nordicum* or *P. verrucosum* OTA biosynthetic genes, genes from *Aspergillus* species were queried instead. As a result, we suggest that genes encoded within cluster 39 (named herein), which covers the entirety of scaffold_112, and *gene_3514* may be involved with OTA production by *P. thymicola* DAOMC 180753. Together, they encode the typical protein domains found in PKS and NRPS enzyme complexes and are homologous to enzymes critical for OTA biosynthesis by *Aspergillus* species. The protein from *P. thymicola* for *gene_4875* (jgi|Penth1|212294), part of cluster 39, showed >70 % homology to an *Aspergillus ochraceus* PKS protein (GenBank AAP32477.1) (O'Callaghan *et al.* 2003) and the *AcOTApks* protein (jgi|Aspca3|173482) from *Aspergillus carbonarius* (Gallo *et al.* 2014). Both these *Aspergillus* genes are directly implicated in OTA biosynthesis, as demonstrated by gene inactivation and expression experiments (Gallo *et al.* 2009; Gallo *et al.* 2012; Gallo *et al.* 2014). The alignment length of the *P. thymicola* and *Aspergillus* genes was short, therefore it did not occur as one of the top BLASTp hits (Suppl. Table 1). However, the portion that aligned coincided with the AT domain of the *AcOTApks* protein, an integral component of any PKS enzyme complex. Aside from biosynthetic genes, cluster 39 includes one regulatory (*gene_4856*) and one transporter (*gene_4860*) gene (Table 3). This transporter gene is weakly homologous to a major facilitator superfamily (MFS) transporter. Geisen *et al.* (2006) reported that the *P. nordicum* gene *otatraPN* has homology with a MFS transporter. A BLAST search querying the UnitProt/Swiss-Prot database did not reveal a halogenase, chloroperoxidase or peptide synthetase genes within cluster 39. Elsewhere in the genome, genes for a potential halogenase and two chloroperoxidases were identified, but they were not located in a cluster with other putative OTA biosynthesis related genes.

Gene_3514 (jgi|Penth1|168734) is a PKS–NRPS hybrid and not part of a gene cluster, although it was located in the middle of a genomic scaffold. This protein was homologous to a *ACpks* protein from *A. carbonarius* (Gallo *et al.* 2009) that was differentially expressed only when OTA was synthesized, although no knockouts were investigated by Gallo *et al.* (2009). Using gene inactivation, Gallo *et al.* (2012) demonstrated that the NRPS gene *AcOTAnrps* is implicated in OTA biosynthesis by *A. carbonarius*. However, the protein of *gene_3514* in *P. thymicola* did not have significant homology with the *AcOTAnrps* protein (Suppl. Table 1).

While *gene_3514* encodes a protein with all the typical NRPS domains, it lacks an ACP domain in the PKS portion; however, *gene_4877* in cluster 39 has this domain. Conversely, genes within cluster 39 do not have the KS domain of a typical PKS while the PKS part of *gene_3514* has a KS domain. Therefore, OTA biosynthesis in *P. thymicola* may fall into one of these occurrences of secondary metabolite genes that are not clustered in a single locus. Unclustered secondary metabolite biosynthetic pathways are rare but do occur. For example, the DHN melanin biosynthesis pathway is not always clustered (Baker 2008; Chiang *et al.* 2011) and the T-toxin cluster in *Cochliobolus heterostrophus* is divided among two clusters (Kodama *et al.* 1999; Turgeon & Baker 2007). In *Fusarium* spp., trichothecene biosynthetic genes are found in multiple loci, with some genes clustered and others existing as isolated genes (reviewed in Kimura *et al.* 2007).

Our analysis of *P. thymicola* DAOMC 180753 genomic data indicates that it possess OTA biosynthetic machinery more similar to that of *Aspergillus* species compared to its sibling species *P. verrucosum* and *P. nordicum*. The major metabolites produced by this species were fumiquinazoline F and alantrypinone, previously identified from the type strain of *P. thymicola* CBS 111225. They are derived from the amino acids alanine and tryptophan (Larsen *et al.* 1998). The amino acid phenylalanine, the non-ribosomal peptide structural component of ochratoxins (Harris & Mantle 2001), was also detected from each of the three *Penicillium* species examined. Fumiquinazoline F was originally reported from an *Aspergillus fumigatus* strain (Takahashi *et al.* 1995). Because *P. thymicola* harbours secondary metabolite biosynthetic capacity for fumiquinazoline F, and this metabolite is absent from *P. verrucosum* or *P. nordicum*, there is some precedence for the notion that some of its secondary metabolite biosynthesis is more similar to *Aspergillus*. Next generation sequencing does have biases and it is possible that an OTA gene cluster in *P. thymicola* occurs in a region that is difficult to sequence, where there is lower coverage or repetitive regions, and therefore remained unassembled. Gene knockout, gene expression and chemical tracing experiments should verify whether the putative genes identified here actually do play a role in OTA biosynthesis in *P. thymicola*. Although a major undertaking, it is only when the intermediate products are detected after inhibition or deletion of a particular gene that functional OTA biosynthesis can be accurately assessed. The detection of citrinin from the *P. verrucosum* culture filtrate extract and not from *P. thymicola* or *P. nordicum* is consistent with the results of Larsen *et al.* (2001a). The alpha pyrone PC-2 was identified from each of the *Penicillium* extracts based on HRMS. *Penicillium thymicola*, *P. nordicum*, and *P. verrucosum* produce this metabolite (Frisvad & Samson 2004), which has been suggested as a biomarker for known OTA-producing *Penicillium* species (Rahbaek *et al.* 2003). An attempt was made to investigate the production of verrucines A and B (*P. verrucosum* metabolites; Larsen *et al.* 1999) and the diketopiperazine lumpidin (*P. nordicum* metabolite; Larsen *et al.* 2001b). Their high resolution precursor ions as well as verrucines product ions reported by Nielsen & Smedsgaard (2003) were queried. None of these compounds were confirmed in the studied extracts. Apart from the production of OTA and OTB by *P. thymicola*, the secondary metabolite profiles reported here agree with the results of Frisvad & Samson (2004).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2016.04.002.

REFERENCES

Abbas A, Coghlan A, O'Callaghan J, Garcia-Estrada C, Martin JF, Dobson AD, 2013. Functional characterization of the polyketide synthase gene required for ochratoxin A biosynthesis in *Penicillium verrucosum*. *International Journal of Food Microbiology* **161**: 172–181.

Bacha N, Atoui A, Mathieu F, Liboz T, Lebrihi A, 2009. *Aspergillus westerdijkiae* polyketide synthase gene "*aoks1*" is involved in the biosynthesis of ochratoxin A. *Fungal Genetics and Biology* **46**: 77–84.

Baker SE, 2008. *Aspergillus* genomics and DHN-melanin conidial pigmentation. In: Varga J, Samson R (eds), *Aspergillus in the Genomics Era*. Wageningen Academic Publishers, Wageningen, pp. 73–85.

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA, 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology* **19**: 455–477.

Barlow S, Bolger M, Pitt JI, Verger P, 2008. Ochratoxin A (addendum). In: *WHO Food Additives Series 59. Safety Evaluation of Certain Contaminants in Food*. World Health Organization, Geneva, pp. 357–429.

Blin K, Medema MH, Kazempour D, Fischbach MA, Breitling R, Takano E, Weber T, 2013. antiSMASH 2.0–a versatile platform for genome mining of secondary metabolite producers. *Nucleic Acids Research* **41**: W204–W212.

Bolger AM, Lohse M, Usadel B, 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**: 2114–2120.

Bondy GS, Caldwell DS, Aziz SA, Coady LC, Armstrong CL, Curran IH, Koffman RL, Kapal K, Lefebvre DE, Mehta R, 2015. Effects of chronic Ochratoxin A exposure on p53 heterozygous and p53 homozygous mice. *Toxicologic Pathology* **43**: 715–729.

Cabañes FJ, Bragulat MR, Castellá G, 2010. Ochratoxin A producing species in the genus *Penicillium*. *Toxins* **2**: 1111–1120.

Chiang YM, Meyer KM, Praseuth M, Baker SE, Bruno KS, Wang CCC, 2011. Characterization of a polyketide synthase in *Aspergillus niger* whose product is a precursor for both dihydroxynaphthalene (DHN) melanin and naphtho-γ-pyrone. *Fungal Genetics and Biology* **48**: 430–437.

Chikhi R, Medvedev P, 2014. Informed and automated k-mer size selection for genome assembly. *Bioinformatics* **30**: 31–37.

Duarte SC, Pena A, Lino CM, 2010. A review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. *Food Microbiology* **27**: 187–198.

el Khoury A, Atoui A, 2010. Ochratoxin a: general overview and actual molecular status. *Toxins* **2**: 461–493.

Frisvad JC, Samson RA, 2004. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*: a guide to identification of food and airborne terverticillate *Penicillia* and their mycotoxins. *Studies in Mycology* **49**: 1–174.

Gallo A, Perrone G, Solfrizzo M, Epifani F, Abbas A, Dobson AD, Mulè G, 2009. Characterisation of a pks gene which is expressed during ochratoxin A production by *Aspergillus carbonarius*. *International Journal of Food Microbiology* **129**: 8–15.

Gallo A, Bruno KS, Solfrizzo M, Perrone G, Mulè G, Visconti A, Baker SE, 2012. New insight into the ochratoxin A biosynthetic pathway through deletion of a nonribosomal peptide synthetase gene in *Aspergillus carbonarius*. *Applied and Environmental Microbiology* **78**: 8208–8218.

Gallo A, Knox BP, Bruno KS, Solfrizzo M, Baker SE, Perrone G, 2014. Identification and characterization of the polyketide synthase involved in ochratoxin A biosynthesis in *Aspergillus carbonarius*. *International Journal of Food Microbiology* **179**: 10–17.

Garcia-Alcalde F, Okonechnikov K, Carbonell J, Cruz LM, Gotz S, Tarazona S, Dopazo J, Meyer TF, Conesa A, 2012. Qualimap: evaluating next-generation sequencing alignment data. *Bioinformatics* **28**: 2678–2679.

Geisen R, Schmidt-Heydt M, Karolewiez A, 2006. A gene cluster of the ochratoxin A biosynthetic genes in *Penicillium*. *Mycotoxin Research* **22**: 134–141.

Grigoriev IV, Martinez DA, Salamov AA, 2006. Fungal genomic annotation. In: Aurora DK, Berka RM, Singh GB (eds), *Applied Mycology and Biotechnology*, vol. 6. Elsevier, Amsterdam, pp. 123–142.

Grigoriev IV, Nikitin R, Haridas S, Kuo A, Ohm R, Otillar R, Riley R, Salamov A, Zhao X, Korzeniewski F, Smirnova T, Nordberg H, Dubchak I, Shabalov I, 2014. MycoCosm portal: gearing up for 1000 fungal genomes. *Nucleic Acids Research* **42**: D699–D704.

Gurevich A, Saveliev V, Vyahhi N, Tesler G, 2013. QUAST: quality assessment tool for genome assemblies. *Bioinformatics* **29**: 1072–1075.

Harris JP, Mantle PG, 2001. Biosynthesis of ochratoxins by *Aspergillus ochraceus*. *Phytochemistry* **58**: 709–716.

IARC (International Agency for Research on Cancer), 1993. Ochratoxin A. In: *IARC monographs on the evaluation of carcinogenic risks to humans*, vol. 56. IARC Press, Lyon, pp. 489–521.

Karolewiez A, Geisen R, 2005. Cloning a part of the ochratoxin A biosynthetic gene cluster of *Penicillium nordicum* and characterization of the ochratoxin polyketide synthase gene. *Systematic and Applied Microbiology* **28**: 588–595.

Keller NP, Turner G, Bennett JW, 2005. Fungal secondary metabolism – from biochemistry to genomics. *Nature Reviews Microbiology* **3**: 937–947.

Kimura M, Tokai T, Takahashi-Ando N, Ohsato S, Fujimura M, 2007. Molecular and genetic studies of *Fusarium* trichothecene biosynthesis: pathways, genes, and evolution. *Bioscience, Biotechnology, and Biochemistry* **71**: 2105–2123.

Kodama M, Rose MS, Yang G, Yun SH, Yoder OC, Turgeon BG, 1999. The translocation-associated tox1 locus of *Cochliobolus heterostrophus* is two genetic elements on two different chromosomes. *Genetics* **151**: 585–596.

Kuiper-Goodman T, Hilts C, Billiard SM, Kiparissis Y, Richard ID, Hayward S, 2010. Health risk assessment of ochratoxin A for all age-sex strata in a market economy. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment* **27**: 212–240.

Langmead B, Salzberg SL, 2012. Fast gapped-read alignment with Bowtie 2. *Nature Methods* **9**: 357–359.

Larsen TO, Frydenvang K, Frisvad JC, Christophersen C, 1998. UV-guided isolation of alantrypinone, a novel *Penicillium* alkaloid. *Journal of Natural Products* **61**: 1154–1157.

Larsen TO, Franzyk H, Jensen SR, 1999. UV-guided isolation of verrucines A and B, novel quinazolines from *Penicillium*

verrucosum structurally related to anacine from *Penicillium aurantiogriseum*. *Journal of Natural Products* **62**: 1578–1580.

Larsen TO, Svendsen A, Smedsgaard J, 2001a. Biochemical characterization of ochratoxin A-producing strains of the genus *Pencillium*. *Applied and Environmental Microbiology* **67**: 3630–3635.

Larsen TO, Petersen BO, Duus JO, 2001b. Lumpidin, a novel biomarker of some ochratoxin A producing *Penicillia*. *Journal of Agricultural and Food Chemistry* **49**: 5081–5084.

Lee HJ, Ryu D, 2015. Significance of Ochratoxin A in Breakfast Cereals from the United States. *Journal of Agricultural and Food Chemistry* **63**: 9404–9409.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 2009. The sequence alignment/map format and SAMtools. *Bioinformatics* **25**: 2078–2079.

Lund F, Frisvad JC, 2003. *Penicillium verrucosum* in wheat and barley indicates presence of ochratoxin A. *Journal of Applied Microbiology* **95**: 1117–1123.

Miller JD, Schaafsma A, Bhatnagar D, Bondy G, Carbone I, Harris L, Harrison G, Munkvold G, Oswald I, Pestka J, Sharpe L, Sumarah M, Tittlemier S, Zhou T, 2014. Mycotoxins that affect the North American agri-food sector: state of the art and directions for the future. *World Mycotoxin Journal* **7**: 63–82.

Nguyen KTN, Ryu D, 2014. Concentration of ochratoxin A in breakfast cereals and snacks consumed in the United States. *Food Control* **40**: 140–144.

Nielsen KF, Smedsgaard J, 2003. Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for dereplication by standardised liquid chromatography–UV–mass spectrometry methodology. *Journal of Chromatography A* **1002**: 111–136.

Nikolenko SI, Korobeynikov AI, Alekseyev MA, 2013. BayesHammer: Bayesian clustering for error correction in single-cell sequencing. *BMC Genomics* **14**: S7.

O'Callaghan J, Caddick MX, Dobson AD, 2003. A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. *Microbiology* **149**: 3485–3491.

Parra G, Bradnam K, Korf I, 2007. CEGMA: a pipeline to accurately annotate core genes in eukaryotic genomes. *Bioinformatics* **23**: 1061–1067.

Rahbaek L, Sperry S, Frisvad JC, Larsen TO, 2003. PC-2, LL-P888gamma and some novel analogue alpha-pyrones from *Penicillium nordicum*, *P. verrucosum* and *P. olsonii*. *Biochemical Systematics and Ecology* **31**: 313–317.

Samson RA, Houbraken J, Kuijpers A, Frank JM, Frisvad JC, 2004. New ochratoxin A or sclerotium producing species in *Aspergillus* section Nigri. *Studies in Mycology* **50**: 45–61.

Schmidt-Heydt M, Baxter E, Geisen R, Magan N, 2007. Physiological relationship between food preservatives, environmental factors, ochratoxin and *otapksPV* gene expression by *Penicillium verrucosum*. *International Journal of Food Microbiology* **119**: 277–283.

Schwarzer D, Finking R, Marahiel MA, 2003. Nonribosomal peptides: from genes to products. *Natural Product Reports* **20**: 275–287.

Sonjak S, Ličen M, Frisvad JC, Gunde-Cimerman N, 2011. Salting of dry-cured meat–A potential cause of contamination with the ochratoxin A-producing species *Penicillium nordicum*. *Food Microbiology* **28**: 1111–1116.

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 Communications in Mass Spectrometry* **20**: 2649–2659.

Takahashi C, Matsushita T, Doi M, Minoura K, Shingu T, Kumeda Y, Numata A, 1995. Fumiquinazolines A-G, novel metabolites of fungus separated from a *Pseudolabrus* marine fish. *Journal of the Chemical Society Perkin Transactions* **1**: 2345–2353.

Turgeon BG, Baker SE, 2007. Genetic and genomic dissection of the *Cochliobolus heterostrophus Tox1* locus controlling biosynthesis of the polyketide virulence factor T-toxin. *Advances in Genetics* **57**: 219–261.

van Egmond HP, Schothorst RC, Jonker MA, 2007. Regulations relating to mycotoxins in food. *Analytical and Bioanalytical Chemistry* **389**: 147–157.

Varga J, Rigo K, Teren J, Mesterhazy A, 2001. Recent advances in ochratoxin research. I. Production, detection and occurrence of ochratoxins. *Cereal Research Communications* **29**: 85–92.

Varga J, Frisvad JC, Kocsubé S, Brankovics B, Tóth B, Szigeti G, Samson RA, 2011. New and revisited species in *Aspergillus* section Nigri. *Studies in Mycology* **69**: 1–17.

Varga E, Glauner T, Berthiller F, Krska R, Schuhmacher R, Sulyok M, 2013. Development and validation of a (semi-) quantitative UHPLC-MS/MS method for the determination of 191 mycotoxins and other fungal metabolites in almonds, hazelnuts, peanuts and pistachios. *Analytical and Bioanalytical Chemistry* **405**: 5087–5104.

Visagie CM, Varga J, Houbraken J, Meijer M, Kocsubé S, Yilmaz N, Fotedar R, Seifert KA, Frisvad JC, Samson RA, 2014a. Ochratoxin production and taxonomy of the yellow aspergilli (*Aspergillus* section Circumdati). *Studies in Mycology* **78**: 1–61.

Visagie CM, Houbraken J, Frisvad JC, Hong SB, Klaassen CHW, Perrone G, Seifert KA, Varga J, Yaguchi T, Samson RA, 2014b. Identification and nomenclature of the genus *Penicillium*. *Studies in Mycology* **78**: 343–371.

Yu JH, Keller N, 2005. Regulation of secondary metabolism in filamentous fungi. *Annual Review of Phytopathology* **43**: 437–458.

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