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Description of an orthologous cluster of ochratoxin A biosynthetic genes in *Aspergillus* and *Penicillium* species. A comparative analysis

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ABSTRACT

Ochratoxin A (OTA) is one of the most important mycotoxins due to its toxic properties and worldwide distribution which is produced by several *Aspergillus* and *Penicillium* species. The knowledge of OTA biosynthetic genes and understanding of the mechanisms involved in their regulation are essential. In this work, we obtained a clear picture of biosynthetic genes organization in the main OTA-producing *Aspergillus* and *Penicillium* species (*A. steynii*, *A. westerdijkiae*, *A. niger*, *A. carbonarius* and *P. nordicum*) using complete genome sequences obtained in this work or previously available on databases. The results revealed a region containing five ORFs which predicted five proteins: halogenase, bZIP transcription factor, cytochrome P450 monooxygenase, non-ribosomal peptide synthetase and polyketide synthase in all the five species. Genetic synteny was conserved in both *Penicillium* and *Aspergillus* species although genomic location seemed to be different since the clusters presented different flanking regions (except for *A. steynii* and *A. westerdijkiae*); these observations support the hypothesis of the orthology of this genomic region and that it might have been acquired by horizontal transfer.

New real-time RT-PCR assays for quantification of the expression of these OTA biosynthetic genes were developed. In all species, the five genes were consistently expressed in OTA-producing strains in permissive conditions. These protocols might favour futures studies on the regulation of biosynthetic genes in order to develop new efficient control methods to avoid OTA entering the food chain.

1. Introduction

Ochratoxin A (OTA) is a toxic fungal secondary metabolite (SM) which contaminates a variety of foodstuffs including cereals, grapes, coffee, nuts and spices, among others, with a worldwide distribution (Malir et al., 2016). The high toxicity of this compound and its ability to accumulate in human and animal organs represent a high risk for food safety (Heussner and Bingle, 2015) and, consequently, its levels are monitored in many countries and are subjected to strict legal regulations for certain agroproducts (Duarte et al., 2010). OTA is produced by several fungal species included in *Aspergillus* and *Penicillium* genera (Wang et al., 2016). *P. nordicum* and *P. verrucosum* are the main *Penicillium* species capable of producing OTA. They are an important source of the toxin in meat products and cereals, respectively, particularly in temperate regions (El Khoury and Atoui, 2010). OTA-producing *Aspergillus* species are included in sections *Circumdati* and *Nigri*. Recent studies combining phylogenetic and SM profile analyses uncovered cryptic and new species, increasing the number of OTA-producing species described. Nowadays, the main OTA-producing species in *Aspergillus* section *Circumdati* are *A. steynii and A. westerdijkiae* (Gil-Serna et al., 2011b) whereas *A. carbonarius*, *A. niger* and *A. welwitschiae* are the most important producers in section *Nigri* (Palumbo and O'Keeffe, 2015; Susca et al., 2016).

The biosynthetic genes involved in SM production are often clustered in fungi (Keller and Hohn, 1997), an organization which facilitates horizontal gene transfer to other species (Wisecaver and Rokas, 2015) as well as coordination of their transcriptional activation and regulation (Shwab and Keller, 2008; Umemura et al., 2015).

Studies on the OTA biosynthetic pathway preclude the introduction of a chlorine atom to ochratoxin β by a chloroperoxidase (CHL) or halogenase (HAL) enzyme (Wang et al., 2016). Geisen et al. (2006) identified a CHL encoding gene in the putative cluster of OTA biosynthesis in *P. nordicum*. Recently, Ferrara et al. (2016) described a new HAL gene in OTA biosynthetic cluster and Gerin et al. (2016) also found a CHL gene up-regulated in OTA permissive conditions in *A. carbonarius* but the sequences do not seem to be homologous to that reported in *P.*

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nordicum.

Currently, the key genes for OTA biosynthesis are known to encode a polyketide synthase (PKS) and a non-ribosomal peptide synthase (NRPS) and, therefore, the research on these genes is far more advanced (Geisen and Schmidt-Heydt, 2009). Up to now, different genes putatively involved in OTA synthesis encoding PKSs and NRPSs have been described in both *Penicillium* and *Aspergillus* species (Abbas et al., 2013; Bacha et al., 2009; Gallo et al., 2012; Gallo et al., 2014; Geisen et al., 2006; Gil-Serna et al., 2015; Karolewiez and Geisen, 2005; O'Callaghan et al., 2003; Schmidt-Heydt et al., 2007; Wang et al., 2015; Zhang et al., 2016). However, PKS and NRPS genes reported in *Penicillium* (*otapksPN* and *npsPN*) seem to be non-homologous to those reported so far in *Aspergillus* species, indicating that they might be genus specific (Wang et al., 2016). Additional genes that have been reported as putatively involved in OTA biosynthesis encoded cytochrome *p*450 monooxygenases (P450) in *A. westerdijkiae* (O'Callaghan et al., 2006) and *A. steynii* (Gil-Serna et al., 2015).

Next generation sequencing has provided a useful tool to unravel the genetic location of the genes involved in mycotoxin biosynthesis. Recently, Ferrara et al. (2016) and Susca et al. (2016) reported that genes involved in OTA biosynthesis were located consecutively in the genome of *Aspergillus* section *Nigri* species. They described a region including a HAL, a transcription factor, a P450, a NRPS and a PKS encoding genes which supplemented the knowledge of the clustered array of a P450, NRPS and PKS encoding genes involved in OTA biosynthesis previously reported in *A. steynii* (Gil-Serna et al., 2015). A similar putative cluster has been recently described in *A. westerdijkiae* genome although the relation of these genes to OTA production has not been evaluated yet (Han et al., 2016). Currently several genome sequences of OTA-producing species are available on databases and they provide the opportunity to compare the organization of those genes previously described within the cluster in the different species and unravel relevant motifs to their function and regulation and develop useful tools for the study of OTA biosynthesis. These aspects are also crucial to develop control strategies to reduce the OTA risk in agrofood products.

The aims of this work were: (i) to obtain a clear picture of the organization of the OTA biosynthetic genes in the main producing species in *Aspergillus* and *Penicillium* (*A. steynii*, *A. westerdijkiae*, *A. niger*, *A. carbonarius* and *P. nordicum*) using complete genome sequences obtained in this work or previously available on databases and (ii) to develop useful real-time RT-PCR assays for quantification of expression of the OTA biosynthetic genes encoding HAL, bZIP, P450, NRPS, PKS proteins in those fungal species and to study their expression in OTA permissive conditions.

2. Materials and methods

2.1. Genome analysis and putative cluster identification

The complete genome sequences of *A. steynii* CBS 112.812 (Baker, 2014), *A. carbonarius* ITEM 5010 (Baker, 2010) and *A. niger* CBS 513.88 (Pel et al., 2007) were available on JGI Genome Portal website whereas the whole genome of *P. nordicum* DAOMC 185683 were obtained from the NCBI database (Nguyen and Seifert, 2015). The genome sequence of *A. westerdijkiae* (type strain CECT 2948) was obtained in this work. Sequences of the OTA biosynthetic cluster described previously in *A. steynii* strain 3.53 (Gil-Serna et al., 2015) were used as reference to retrieve putative OTA biosynthetic clusters in the genomes of the species above mentioned.

Bioinformatics analyses were performed using Unipro UGENE 1.25.0 including identity calculations and protein sequence prediction (Okonechnikov et al., 2012). Function prediction and domain analysis were carried out using PFAM 31.0 software available on the EMBL webpage (http://pfam.xfam.org).

2.2. Genome sequencing of Aspergillus westerdijkiae type strain

The genome sequence of *A. westerdijkiae* type strain CECT 2948 was obtained by next generation sequencing in the lllumina HiSeq platform (Applied Biosystems, USA) (Stab-Vida, Portugal), using 100 bp paired-end sequencing reads. The analysis of the generated raw sequence data was carried out using CLC Genomics Workbench 9.0 and the raw sequence data of the sample were *de novo* assembled using an algorithm based on Bruijn graphs. The average contig length obtained was 92,477 and a genome size of 36.34 Mb was found.

Genomic DNA was isolated from 3-day-old cultures in potato-dextrose broth (Pronadisa, Spain) using the DNeasy Plant Mini Kit (QIAgen, Spain) following manufacturer's instructions. The DNA sample was used for library construction using the TruSeq DNA Whole genome library preparation kit (Illumina, USA).

2.3. Phylogenetic analysis

DNA sequences were aligned using Clustal W algorithm and, subsequently, a phylogeny was inferred by using the Maximum Parsimony method. Phylogenetic analysis involved five nucleotide sequences corresponding to the complete cluster in *A. niger*, *A. steynii*, *A. westerdijkiae* and *P. nordicum*. Additionally, a similar analysis was performed excluding introns and intergenic regions and only encoding regions were used. Consistency index was calculated for parsimony-informative sites. The most parsimonious tree was obtained using the Close-Neighbour-Interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (100 replicates). All positions containing gaps and missing data were eliminated. There were a total of 17,296 positions in the final dataset for the complete clusters analysis and 15,751 positions if only cDNA sequences were included. All evolutionary analyses were conducted in MEGA 5 (Tamura et al., 2011).

2.4. Strains and culture conditions

All experiments were performed using both an OTA-producing strain (P) and a negative control strain (NP), unable to produce OTA or producing it at very low levels at these conditions. Two replicates for each condition were evaluated. Non-producing strains of *A. westerdijkiae*, *A. steynii* and *A. carbonarius* presented an intact cluster containing the five genes since amplification bands of the correct size were obtained by PCR using genomic DNA. Non-producing strain of *A. niger* did not possess the OTA cluster in its genome and only a short part of the 3′ end of the PKS encoding gene is present in fungal genome; therefore, no amplification bands were found by PCR even using genomic DNA. In the case of *P. nordicum*, it was not possible to find a strain defective in OTA production in CYA medium and, therefore, the composition of culture medium was modified to achieve differences in OTA production between the isolates tested.

The strains of *A. steynii* 3.53 (P) and CBS 112813 (NP), *A. westerdijkiae* strains CECT 2948 (P) and 3.38 (NP), *A. niger* strains 3.39 (P) and NIG1 (NP) and *A. carbonarius* strains 350 (P) and 265 (NP) were cultured in plates containing CYA medium (45.4 g/L Czapek-Dox Modified Agar, 5 g/L yeast extract) for 5 days. *P. nordicum* strains CBS 110.769 (P) and CBS 323.912 (NP, low producer) were cultured in YES medium with low sucrose concentration (20 g/L sucrose, 20 g/L yeast extract and 20 g/L bacteriological agar) for 7 days to achieve the highest differences in OTA production by both isolates following the recommendations made by Bernáldez-Rey (2016).

Sterile cellophane membranes (BioRad, Spain) were laid on the plates before fungal inoculation to facilitate mycelia removal after incubation. Spore suspensions (1.5 µl, 10^7 spores/mL) were located in the centre of the plates and the incubation was performed at 28 °C. At the end of incubation period, the cellophane with fungal mycelium was removed from the plate and frozen at − 80 °C for RNA extraction. OTA