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Ochratoxin A biosynthetic genes in *Aspergillus ochraceus* are differentially regulated by pH and nutritional stimuli

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Abstract

Expression of the polyketide synthase (*pks*) gene which is involved in ochratoxin A (OTA) biosynthesis in *Aspergillus ochraceus* is linked to production of the mycotoxin, with high levels of *pks* mRNA accumulation occurring in cultures producing OTA, as assessed by real-time reverse transcription (RT)-PCR. OTA production is regulated by nutrient availability, with supplementation of OTA restrictive potato dextrose broth with yeast extract resulting in a 39-fold increase in production of the mycotoxin. This effect appears to be mediated at the level of gene transcription as there is a concomitant increase in *pks* mRNA accumulation. OTA production is also strongly influenced by culture pH with large amounts of OTA being produced at pH values <7.0 with reduced amounts being produced at higher pH values. *pks* transcript levels again mirrored the OTA production profile observed at the different pH values. The transcription of two putative p450 type monooxygenase genes, namely p450-H11 and p450-B03 genes closely mirrored that of the *pks* gene under all growth conditions tested, suggesting their involvement together with the *pks* in OTA biosynthesis. The expression profile of the p450-B03 gene in particular is very similar to that of the *pks* gene, indicating that this gene may be clustered with the *pks* as part of the OTA biosynthetic gene cluster.

Keywords: Polyketide synthase; Cytochrome P450 monooxygenase; Ochratoxin A; *Aspergillus ochraceus*

1. Introduction

Ochratoxin A (OTA) is a mycotoxin consisting of a polyketide derived from a dihydroiso-coumarin moiety linked through the 12-carboxyl group to phenylalanine, via an amide linkage. It is a nephrotoxin which also displays hepatotoxic, teratogenic, and immunosuppressive properties; and has been classified by The International Agency for Research on Cancer as a possible human carcinogen (category 2B) (Kuiper-Goodman and Scott, 1989; Petzinger and Ziegler, 2000). OTA is also known to impede cellular physiology in humans, primarily by disturbing phenylalanine metabolism an effect which appears to be mediated by the inhibition of enzymes involved in phenylalanine tRNA synthesis (Marquardt and Frohlich, 1992). OTA is often cited as a causative factor in the human disease balkan endemic nephropathy (BEN), a chronic nephritis which occurs in countries bordering the river Danube in Europe, but a direct connection has not been definitively proven (Tatu et al., 1998). Indeed recent reports suggest the likely involvement of additional genetic components in BEN (Atanasova et al., 2005).

OTA is a common contaminant of grains such as barley, corn, rye, wheat, and oats, with cereal-based products typically accounting for 50–80% of the average consumer intake of the mycotoxin (Jorgensen and Jacobsen, 2002). OTA has also been reported in other plant products including coffee beans, spices, nuts, olives, grapes, beans, and figs

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(Battilani et al., 2003; Batista et al., 2003; Bayman et al., 2002; Jorgensen, 1998). There is a risk to human health not only through the intake of contaminated foods of vegetable origin, but also through foods of animal origin with OTA being reported in animal derived food products, such as poultry and pork meat and in offal and sausages containing pork blood, due to the feeding of mould contaminated fodder to animals (Gareis and Scheurer, 2000; Petzinger and Weidenbach, 2002). In addition OTA can survive many typical food-processing procedures, and has been reported in bread made from contaminated wheat (Scudamore et al., 2003) and in beers (Odhav and Naicker, 2002). In humans OTA displays unusual toxicokinetics, with a half-life in blood of 35 days after oral ingestion (Schlatter et al., 1996) and thus not surprisingly perhaps has been detected in human blood samples (Thuvander et al., 2001) as well as in human breast milk (Skaug et al., 2001).

OTA is produced by both *Penicillium verrucosum* and *Aspergillus ochraceus*, with the latter being the most common OTA producer in tropical regions while the former predominates in temperate regions such as eastern and north eastern Europe, Canada, and parts of South America (Pitt, 2000). Other OTA producing strains include *Aspergillus carbonarius*, which can affect wine grapes, sometimes resulting in the contamination of wines made from these grapes (Bau et al., 2005) and *Aspergillus alliaceus* which typically contaminates nuts and figs (Bayman et al., 2002). *Penicillium* strains that produce OTA have recently been reclassified as either *P. verrucosum* or *Penicillium nordicum* (Castella et al., 2002). These two species appear to differ in the habitats they occupy, with *P. verrucosum* being almost exclusively a storage fungus which contaminates cereals while *P. nordicum* is more adapted to food environments such as cheeses and fermented meats (Lund and Frisvad, 2003). Unlike *Penicillium*, many different *Aspergillus* species are known to produce OTA including those from section *Flavi*, section *Nigri*, and section *Aspergillus* (Abarca et al., 1994; Dalcero et al., 2002; Teren et al., 1996; Varga et al., 2003).

The OTA biosynthetic pathway has not yet been elucidated in any fungal species; but it is believed that the isocoumarin group is a pentaketide formed from acetate and malonate possibly via a polyketide synthesis pathway (Moss, 1998). The heterocyclic portion of OTA is structurally similar to mellein, a common fungal metabolite. A C1 unit is added to a carboxyl group at C-8, while a chlorine atom is also incorporated, potentially through the action of a chloroperoxidase. L-Phenylalanine which is derived from the shikimic acid pathway is linked through the additional carboxyl group (Edwards et al., 2002; Moss, 1998). Recently there have been a number of studies aimed at identifying genes that are essential for OTA biosynthesis. In an attempt to elucidate the OTA biosynthetic pathway we have previously cloned part of the polyketide synthase (*pks*) gene required for OTA biosynthesis in *A. ochraceus* (O'Callaghan et al., 2003). In addition, a polyketide synthase gene *otapks*PN, from *P. nordicum* that is essential for OTA biosynthesis has been reported by Karolewiez and Geisen (2005). Expression of this *otapks*PN gene in *P. nordicum* has been reported to be lower under acidic conditions below pH 5 (Geisen, 2004). In this paper we report that expression of the *pks* gene in *A. ochraceus* is differentially regulated and correlates well with OTA biosynthesis. In addition, expression of the *pks* gene and production of OTA is regulated not only by nutrient availability but also by the pH of the growth medium. Finally we report on the co-expression of two putative p450 monooxygenase genes together with the *pks* gene, under different physiological conditions; indicating a possible role for these genes in OTA biosynthesis.

2. Materials and methods

2.1. Fungal strain and growth conditions

Aspergillus ochraceus HP was obtained from the University College Cork culture collection and has previously been described (O'Callaghan et al., 2003). The culture was routinely subcultured on potato dextrose agar (Difco) and incubated at 25 °C. Stock cultures were stored at 4 °C on potato dextrose agar slants and long term stocks were stored at −70 °C in glycerol. For experiments on OTA production *A. ochraceus* was grown in Czapek-Dox broth with added yeast extract (0.5% w/v) (CYB), Czapek-Dox broth with casamino acids (acid hydrolysed, vitamin free) (0.5 g/L) and urea (3 g/L) (MCB), and potato dextrose broth (PDB). The medium was further modified to adjust the growth conditions; to adjust pH, either CH_3COOH or NaOH was added. The nitrogen source was adjusted by adding either yeast extract (0.5% w/v) or casamino acids (0.5 g/L) to the potato dextrose broth.

2.2. Measurement of OTA production by A. ochraceus

Ochratoxin A production was measured by HPLC as described by Sibanda et al. (2002) on a Beckman System Gold™ HPLC apparatus using a Beckman Ultrasphere ODS 4.6 × 250 mm column. The mobile phase was acetonitrile:water:acetic acid 99:99:2 and OTA was detected using a Merck-Hitachi fluorescence detector with an excitation wavelength of 333 nm and an emission wavelength of 460 nm. Media samples from *A. ochraceus* cultures were centrifuged to remove mycelium and mixed with an equal volume of HPLC mobile phase prior to injection of 50 µl into the HPLC apparatus.

2.3. Extraction of RNA and preparation of cDNA

Briefly, mycelia were harvested at the appropriate time, weighed, and frozen immediately at −70 °C until the RNA was extracted. To extract the RNA the mycelia were ground in liquid nitrogen in a pre-cooled mortar and pestle. Total RNA was extracted from the ground mycelia using the Qiagen RNeasy plant mini-kit following the manufacturer's instructions. The extracted RNA was treated with