A polyketide synthase gene required for ochratoxin A biosynthesis in Aspergillus ochraceus

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Ochratoxin A is an important nephrotoxic and nephrocarcinogenic mycotoxin, produced by Aspergillus ochraceus as a polyketide-derived secondary metabolite. A portion of a putative polyketide synthase gene (pks) involved in the biosynthesis of this mycotoxin was cloned by using a suppression subtractive hybridization PCR-based approach. The predicted amino acid sequence of the 1·4 kb clone shared 28–35 % identity to acyl transferase regions from fungal polyketide synthases found in the databases. Based on reverse transcription PCR studies, the pks gene is expressed only under ochratoxin A permissive conditions and only during the early stages of the mycotoxin synthesis. A mutant in which the pks gene has been interrupted cannot synthesize ochratoxin A. This report is the first of the cloning and characterization of a gene involved in ochratoxin A biosynthesis.

INTRODUCTION

Ochratoxin A (OTA) is a fungal secondary metabolite consisting of a chlorinated isocoumarin derivative linked to L-phenylalanine. OTA was first discovered in 1965 as a fungal metabolite that was toxic to animals (van der Merwe et al., 1965). It is a potent teratogen and hepatotoxin and has been classified as a possible carcinogen for humans (Lindsey, 2002; Petziner & Ziegler, 2000) because it forms DNA adducts (Dai et al., 2003). There is a correlation between chronic interstitial nephritis and high exposure to OTA (Maaroufi et al., 1995), and an immunomodulatory effect of OTA on a human monocyte/macrophage cell line has been established (Müller et al., 2003). OTA may cause the human disease Balkan Endemic nephropathy, but this connection has not yet been proven. Tolerance intake levels have been estimated at 100 ng (kg body weight)$^{-1}$ week$^{-1}$ (Joint WHO/FAO Committee on Food Additives, JECFA) and 1·5–5·7 ng (kg body weight)$^{-1}$ day$^{-1}$ (Canada) to not more than 5 ng (kg body weight)$^{-1}$ day$^{-1}$ (European Commission) (Walker, 2002).

OTA is produced by Aspergillus ochraceus and Penicillium verrucosum, with the latter being the most common OTA producer in temperate regions such as northern Europe and Canada and the former is more common in warmer climates (Pitt, 2000). Penicillium spp. producing OTA are now classified as either P. verrucosum or Penicillium nordicum (Castella et al., 2002). Other Aspergillus spp. known to produce OTA include Aspergillus auricomus, Aspergillus melleus, Aspergillus ostiatus, Aspergillus petrakii, Aspergillus sclerotiorum and Aspergillus sulfureus (in the A. ochraceus group); Aspergillus alliaceus and Aspergillus albertainensis (in section Flavi); Aspergillus carbonarius and Aspergillus niger (in section Nigri); and Aspergillus glaucus (in section Aspergillus) (Abarca et al., 2001; Bayman et al., 2002; Dalcero et al., 2002). Few of these species are thought to contaminate foods or animal feeds with OTA, but the toxin has been detected in animal feed (Dalcero et al., 2002). The screening of A. niger strains currently used in biotechnological applications for their ability to produce OTA has also been recommended (Schuster et al., 2002).

OTA has been detected in food products such as wine, beer, grape juice, dried fruit, meat, figs, coffee and cereals (Abarca et al., 1994; Bayman et al., 2002; Cabanes et al., 2002; Creppy, 2002; Gareis & Scheurer, 2000; Hussein & Brasel, 2001; Stefanaki et al., 2003; Taniwaki et al., 2003; Visconti et al., 2000). Cereals, or cereal-based products, normally account for 50–80 % of average consumer intake of OTA (Jørgensen & Jacobsen, 2002); consequently, prevention of OTA formation by fungi in cereals would significantly reduce the overall levels of human exposure.

The biosynthetic pathway for OTA has not yet been completely established; however, the isocoumarin group is a pentaketide skeleton formed from acetate and malonate via a polyketide synthesis pathway with the L-phenylalanine being derived from the shikimic acid pathway (Moss, 1996,
interrupted lose their ability to produce the mycotoxin. A. ochraceus strains of pks biosynthesis that was isolated using a suppression sub-
tractive hybridization PCR (SSH-PCR)-based technique (Diatchenko et al., 1996). We also clearly demonstrate that this pks gene is involved in OTA biosynthesis since mutant strains of A. ochraceus in which the pks gene has been interrupted lose their ability to produce the mycotoxin.

METHODS

Fungal strain and culture conditions. A. ochraceus (HP99) was obtained from the University College Cork Culture Collection. The fungus was grown on either yeast extract sucrose (YES) or Czapek-Dox medium containing yeast extract and Casamino acids (MC) for permissive growth, i.e. OTA produced. The fungus was grown on potato dextrose agar (PDA) or malt extract medium (ME) for restrictive growth, i.e. no OTA produced.

RNA preparation and cDNA synthesis. Mycelia were stored frozen at −70 °C prior to extraction of total RNA. RNA was extracted by using the RNeasy plant mini-kit (Qiagen) and frozen mycelia that had been ground to a fine powder in liquid nitrogen, with a mortar and pestle. The extracted RNA was treated with DNase I (Roche) to remove contaminating DNA and stored at −70 °C until used. cDNA was synthesized from each sample with a SMART cDNA synthesis kit (Clontech Laboratories).

SSH-PCR. This was performed with a PCR-Select kit (Clontech) as specified by the manufacturer. The permissive cDNA served as the tester DNA and the restrictive cDNA as the driver DNA. All PCR amplifications during the cDNA synthesis and SSH-PCR were performed with an Advantage-2 PCR kit (Clontech). The resulting cDNAs were cloned into the pGEM-T easy vector (Promega) and the ligation mixtures were transformed into Escherichia coli TOP10 cells (Invitrogen). The transformants obtained were replica plated onto Luria-Bertani agar (96 per 155 mm Petri dish).

Screening for up-regulated clones. Nested primers from the SSH-PCR kit were used to amplify the cloned insert and the PCR products were transferred to duplicate membrane filters and cross-linked following exposure to UV light (Stratalinker; Stratagene).

Up-regulated clones were identified in Southern hybridizations to Escherichia coli /T10 cells (Invitrogen). The transformants obtained were replica plated onto Luria-Bertani agar (96 per 155 mm Petri dish).

DNA sequencing. Clones to be sequenced were grown in 96-well microtitre plates at 37 °C for 24 h. Glycerol was added to a concentration of 40% (v/v) and the plates were frozen at −70 °C. All DNA preparation and sequencing reactions were performed by Lark Technologies (Essex, UK). Nucleotide and protein sequences of each clone were compared to the NCBI protein databases by using the BLAST-x algorithm. A 408 bp clone with a deduced amino acid sequence that shared a high degree of similarity to a number of polyketide synthase (PKS) proteins was selected for further study.

Cloning of DNA sequences flanking the cloned cDNA. Genomic DNA flanking the 408 bp SSH-PCR clone was cloned by using a single-specific primer-PCR (SSP-PCR)-based approach as described previously (Shyamala & Ames, 1989). A. ochraceus genomic DNA was digested with various restriction enzymes and the fragments were ligated into the appropriate site in the pUC18 vector. The ligation mixture served as a template for PCRs in which a primer specific to the end regions of the pks gene sequence was used with a primer specific to the pUC18 vector to amplify the inserted sequences. A pair of PCR primers (PKS4-KOF and PKS4-KOR; Table 1) was designed to amplify a 1·4 kb fragment of the completed sequence. These primers were used to amplify this sequence from A. ochraceus genomic DNA and the PCR product was cloned into the pGEM-T vector (Promega), generating pAOPKS-J4.

RT-PCR. cDNA was synthesized from mycelia growing in permissive and restrictive media using Expand reverse transcriptase and random hexamer primers (Roche) (Soden & Dobson, 2001). Aliquots of the cDNA synthesis reactions were used as templates for a PCR amplification with primers specific to the cloned gene sequence (2BI1-BF and 2BI1-VR; Table 1). As a control, primers specific for the A. ochraceus glyceraldehyde-3-phosphate dehydrogenase gene (G3PDH-F and G3PDH-R; Table 1) were used to monitor expression of this constitutively expressed gene. All RNA samples were checked for DNA contamination prior to use in RT reactions by using an aliquot as a template in PCRs with the primers G3PDH-F and G3PDH-R. The G3PDH primer pair also spans an intron, the product size from genomic DNA was 800 bp while that from cDNA was 600 bp; the size difference in the products allows identification of PCR products from genomic DNA contamination.

OTA mutant construction. The pks gene sequence was disrupted by inserting the E. coli hygromycin B phosphotransferase gene (hph) flanked by the Aspergillus nidulans trpC promoter and terminator sequences (Cullen et al., 1987) from plasmid pD21 (Tang et al., 1992). The ends of the excised fragment were converted to blunt ends by treatment with the Klenow fragment of DNA polymerase (Promega) and the fragment was ligated into a Smal site in the pks portion of pAOPKS-J4 to produce plasmid pAOPKS-J5 (Fig. 3a). A. ochraceus protoplasts were prepared and transformed with pAOPKS-J5, as described previously (Tilburn et al., 1995) for A. nidulans. Hygromycin-resistant transformants were selected on A. nidulans regeneration medium supplemented with 200 μg hygromycin B ml−1 (Calbiochem). For the preparation of protoplasts and selection of transformants A. ochraceus was incubated at 30 °C, except that immediately after transformation the regeneration plates were held at 20 °C for 24 h. Approximately 120 transformants were

Table 1. PCR primers used in this work

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3PDH-F</td>
<td>CGGCCTTGCTGTGTTG</td>
</tr>
<tr>
<td>G3PDH-R</td>
<td>TGAGAGGAGGAGTATTG</td>
</tr>
<tr>
<td>HPH-1F</td>
<td>CGGGGGAATGAGAAGTAAAGG</td>
</tr>
<tr>
<td>HPH-1R</td>
<td>GAACCCGCTCGTCTGGTAAG</td>
</tr>
<tr>
<td>PKS4-KOF</td>
<td>AGTGGAGGCAACACAGTGT</td>
</tr>
<tr>
<td>PKS4-KOR</td>
<td>GGGGCGGTATATCGTCCTGT</td>
</tr>
<tr>
<td>PKS-SSP1</td>
<td>ATGGTGGTGGAGGCGAGTAGAGAAG</td>
</tr>
<tr>
<td>PKS-SSP2</td>
<td>TTGTGACCTCTTATGGCCATGTT</td>
</tr>
<tr>
<td>PUC18-1F</td>
<td>CGGCGGTCGCTTCGTTTG</td>
</tr>
<tr>
<td>PUC18-1R</td>
<td>CGGGGCTGCTTCGTTTG</td>
</tr>
</tbody>
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obtained per microgram of plasmid DNA. Putative transformants were transferred to hygromycin-supplemented PDA for purification prior to screening for OTA production.

**Screening of transformants for OTA-negative mutants.** Initially, greater than 150 transformants were screened for OTA production on coconut agar medium (Heenan et al., 1998). Five transformants and a wild-type strain were plated per plate on coconut agar and incubated at 23 °C for 5 days. The plates were illuminated with UV light and transformants without a blue fluorescent halo were selected as atoxigenic. Putative atoxigenic transformants were selected for a secondary screening that was carried out as follows. A plug of agar was removed from the coconut agar plate at the edge of each colony. Each plug was placed in a microfuge tube containing 500 μl of chloroform and 75 μl of 1 M phosphoric acid. The tube contents were mixed vigorously using a vortex mixer and centrifuged at 14 000 g for 2 min to separate the phases. An aliquot (250 μl) of the organic phase was removed and the chloroform evaporated in a fume hood at 55 °C. The residue was resuspended in 20 μl of methanol and applied to an activated silica TLC plate (Merck). Two samples of pure OTA (Sigma) were applied to each plate as a reference. The chromatograms were run in toluene/ethyl acetate/formic acid (5:4:1) (Bullerman, 1987), visualized under ultraviolet light and photographed.

**Confirmation of disruption of the pks gene by Southern blot.** Genomic DNA from both the wild-type and the mutant strain was digested with SmaI, electrophoresed on a 0.8 % agarose gel and transferred to duplicate nitrocellulose membranes (Southern, 1992). One membrane was hybridized to a pks gene probe amplified with primers PKS4-KOF and PKS4-KOR by PCR from the genome of the wild-type strain, the other was hybridized to a hygromycin gene probe amplified with primers HPH-1F and HPH-1R from pID21 (Table 1). Both probes were labelled with [α-32P]dATP by priming with random hexanucleotides (Prime-a-Gene; Promega). Hybridizations were performed at 55 °C for 12 h in hybridization buffer (0.5 M sodium phosphate/5% SDS, pH 7.0), washed once in 2× SSC/0.1% SDS and twice in 0-2× SSC/0.1% SDS before autoradiography on KODAK MR film.

**HPLC analysis of OTA production.** The extraction procedure for the HPLC assay was identical to that outlined for the TLC assays except that the dried extract was dissolved in 100 μl of methanol rather than in 20 μl. The HPLC method was as described by Sibanda et al. (2002) using a Beckman System Gold HPLC apparatus and a Beckman Ultrasphere C18 (250 × 4.6 mm, 5 μM) reversed-phase column. An aliquot (50 μl) of sample was injected using a Beckman 508 autosampler, the mobile phase was acetonitrile/water/ acetic acid (99:99:2) at a flow rate of 1.0 ml min⁻¹ and OTA was detected by UV absorbance at 330 nm on a Beckman 166 UV-VIS detector. Under these conditions OTA eluted from the column at 9-15 min.

**RESULTS**

**SSH-PCR**

Following the cloning step, several thousand white ampicillin-resistant colonies were obtained from the transformation, of which 1440 were replica plated onto LB agar plates and of these 500 were screened for differential expression. In all, 230 (46 %) of the clones hybridized only to the restrictive cDNA probes and were presumed to represent genes expressed only under OTA permissive conditions (Fig. 1). One hundred and fifty of these clones were sequenced and six of the sequenced clones had sequences that displayed identity at the amino acid level to fungal enzymes that had previously been shown to be involved in mycotoxin production. These included clones which displayed 34, 25 and 46 % similarity, respectively, to a HC toxin synthase from Cochliobolus heterostrophus, a trichodiene oxygenase from Fusarium sporotrichoides and an avarantin oxidoreductase from Aspergillus parasiticus. One 408 bp SSH clone displayed 28-35 % identity at the amino acid level to fungal PKSs from C. heterostrophus, Aspergillus terreus and Gibberella moniliformis, with the closest match being with C. heterostrophus (Yang et al., 1996). At the nucleotide level there was little or no similarity to other pks gene sequences. Subsequent cloning of the flanking regions by single-specific primer-PCR (SSP-PCR) added approximately 1 kb to the initial fragment. The deduced amino acid sequence displayed a considerable degree of identity (31 %) with an acyl transferase domain commonly found in PKS proteins (Fig. 2). The presence of the cloned DNA in the genome of A. ochraceus was confirmed in Southern blots with the pks gene as a probe (Fig. 3).

**Expression of the pks gene during OTA production**

In the permissive medium, OTA production was observed from day 6 onwards, but no OTA was ever detected in the restrictive medium (Fig. 4c). The pks gene was expressed in permissive medium on days 4 and 5 (Fig. 4b), but was never detected in mycelium harvested from the restrictive medium (Fig. 4b). The integrity of the RT-PCR assay was confirmed by measuring the expression of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene, which was
constant at all time points under both permissive and restrictive growth conditions (Fig. 4a).

**Construction of a pks-negative mutant in A. ochraceus**

Two hundred and sixteen transformants were obtained following transformation of *A. ochraceus* with AOPKS-J5. Seven putative atoxigenic colonies were identified based on

**Fig. 2.** Alignment of the deduced amino acid sequence of the *A. ochraceus* PKS with acyltransferase regions from other fungal PKS proteins. Boxed residues are identical in all five proteins, while those in bold are identical in a majority (at least three out of five) of the sequences.

**Fig. 3.** (a) Disruption of the pks gene. (b) Hybridization of pks and hph gene probes to genomic DNA from the NUIC118 mutant and wild-type strains. Lanes 1 and 1' contain *A. ochraceus* genomic DNA digested with Smal and probed with pks and hph probes, respectively. Lanes 2 and 2' contain *A. ochraceus* NUIC118 mutant genomic DNA digested with Smal and probed with pks and hph probes, respectively.

**Fig. 4.** (a) RT-PCR analysis of glyceraldehyde-3-phosphate dehydrogenase gene expression in mycelium from *A. ochraceus* grown in both OTA permissive and restrictive medium, with samples taken from day 4 to day 10. (b) RT-PCR analysis of pks gene transcripts under the same conditions. (c) TLC analysis of OTA production from extracts of the culture medium, corresponding to the equivalent time points; S is an OTA standard.
the absence of a blue halo around the colonies, which is clearly visible in the wild-type A. ochraceus strain (Fig. 5a). None of the putative atoxigenic colonies produced OTA based on TLC analyses (Fig. 5b). The inability of transformant NUIC118 to produce OTA was confirmed by HPLC analysis of culture extracts and comparison to authentic standards of OTA (Fig. 6).

The atoxigenic strain A. ochraceus NUIC118 was selected for further study. Ligation of the hygromycin-resistance cassette into the pks gene of NUIC118 destroyed the SmaI site in the wild-type pks gene. Destruction of the SmaI site coupled with integration of the 2·2 kb hph cassette has the effect of creating a SmaI fragment in the mutant that is considerably larger than in the wild-type. The SmaI fragment to which the pks probe bound was larger than that in the wild-type and was identical in size to the fragment to which the hph probe bound — this confirms that the hph cassette had integrated at the pks gene locus (Fig. 3). Two other atoxigenic mutants, NUIC33 and NUIC107, displayed the same hybridization pattern to the hph and pks probes as NUIC118.

**DISCUSSION**

The steps in the biosynthesis of OTA have not yet been established and the proposed pathway (Moss, 1998) is hypothetical. The isocoumarin moiety of OTA is probably derived from a pentaketide skeleton formed from acetate and malonate via a polyketide type pathway, and the L-phenylalanine is probably from the shikimic acid pathway (Moss, 1996; Moss, 1998). Since OTA has a polyketide backbone, a PKS is probably involved in its synthesis.

We have already cloned three different pks genes from A. ochraceus, which are actively transcribed but are not involved in OTA biosynthesis (Edwards et al., 2002). In this study, we targeted genes that were differentially expressed in A. ochraceus under OTA permissive conditions using an SSH-PCR-based approach. Of 150 differentially expressed genes, the products of six were similar ( > 50 % identity at the amino acid level) to fungal enzymes known to be involved in mycotoxin production, with one of these encoding a PKS.

The gene we sequenced includes a highly conserved acyl transferase region typical of PKS proteins, with this region displaying a high level of similarity (55–60 % at the amino acid level) to other fungal PKS acyl transferases. PKSs and fatty acid synthases (FASs) contain an acyl transferase domain. The primary reaction in both cases is condensation of short-chain fatty acids to form β-ketoacyl thioesters. It is in the subsequent reactions that the PKSs and FASs diverge. The deduced amino acid sequence of our gene is most similar to PKS proteins involved in the biosynthesis of mycotoxins and other bioactive compounds. For example, the PKS proteins with the highest degree of similarity to the A. ochraceus PKS were from C. heterostrophus (T-toxin) (Yang et al., 1996), A. terreus (lovastatin) (Hendrickson et al., 1999), G. moniliformis (fumonisin biosynthesis) (Proctor et al., 2003) and Penicillium citrinum (compactin) (Abe et al., 2002).

The pks gene was not expressed when A. ochraceus was grown on the OTA restrictive medium, but was expressed in permissive medium. Thus, the expression of this pks gene in A. ochraceus is similar to the expression of other PKS-encoding genes such as the pksP1 gene from A. parasiticus and the stcA gene from A. nidulans, which are also differentially regulated under different physiological conditions (Feng & Leonard, 1995). Expression of the pks gene appeared strongest during days 4 and 5 of growth in the
permissive medium and occurred to a much lesser degree at later time points. This expression appeared to correlate with OTA production as assessed by TLC analyses of fungal cultures.

We knocked out the pks gene in A. ochraceus through insertional inactivation with the E. coli hygromycin B phosphotransferase gene (hph). The A. ochraceus NUIC118 mutant no longer produces OTA (Fig. 6), indicating a functional role for the pks gene in OTA biosynthesis.

The cloning of the pks gene should allow us to identify key physiological parameters affecting OTA production in A. ochraceus and in other aspergilli, and allow us to potentially clone OTA biosynthetic gene homologues in P. verrucosum. As genes for secondary metabolite production are often arranged in clusters it will also allow the cloning of other genes involved in OTA biosynthesis from these fungi, and the elucidation of the OTA biosynthetic pathway in both A. ochraceus and P. verrucosum. Continued molecular studies on these fungi should provide information that will help in the development of new strategies for controlling OTA contamination of foods.

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