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# Characterisation of a *pks* gene which is expressed during ochratoxin A production by *Aspergillus carbonarius*

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## Abstract

*Aspergillus carbonarius* is considered the main fungus responsible for ochratoxin A (OTA) contamination in grapes. OTA is a potent nephrotoxin and a possible human carcinogen with a polyketide derived structure. Fungal polyketide synthases (PKSs) have recently been demonstrated to be involved in OTA biosynthesis in both *Penicillium* and *Aspergillus* species. We report here on the identification and characterisation of part of a novel polyketide synthase gene, *ACpks* from *A. carbonarius*. The sequence appears to encode conserved ketosynthase and acyl transferase domains, which are characteristic of previously characterised PKS enzymes. Expression of the *ACpks* gene is differentially regulated, with transcription being observed when the fungus was grown on synthetic grape medium and on OTA permissive medium (MM) whereas no transcription was detected when the fungus was grown on OTA restrictive medium (YES). *ACpks* expression was also observed when *A. carbonarius* was grown at low pH, with concomitant increases in OTA production. This

correlation between *ACpks* gene expression and OTA production suggests the likely involvement for the product of this gene in ochratoxin A biosynthesis in the fungus. From a preliminary screening of *Aspergillus* isolates with *ACpks* specific primers, *ACpks* gene homologues appear to be the present in *A. sclerotioniger* and *A. ibericus*, two species of section *Nigri* which are closely related to *A. carbonarius*.

*Keywords: Aspergillus carbonarius; ochratoxin A; polyketide synthase genes; gene expression; grapes*

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## Introduction

Ochratoxin A (OTA) is a mycotoxin which is produced by several species of *Aspergillus* and *Penicillium* naturally occurring in a variety of food commodities prior to harvest or more commonly during storage. OTA is a potent nephrotoxin, with the degree of renal injury observed, depending on both toxin dose and exposure time. OTA also displays hepatotoxic, teratogenic and immunosuppressive properties and has been reported to act as a potent renal carcinogen in experimental laboratory animals (Castegnaro and Wild, 1995; Creppy, 2002). OTA exposure has also been linked to a fatal human kidney disease called endemic Balkan nephropathy (BEN), which is characterized by an increased incidence of tumours of the urinary tract (Vrabcheva et al., 2004). OTA occurrence in stored cereals and in proteinaceous foods such as cheese and fermented meats is primarily caused by *P. verrucosum* and *P. nordicum*, while several species of the genus *Aspergillus* are responsible for OTA contamination in products such as coffee, raisins, grape juices, spices and wines (Abarca et al., 2001; Pitt, 2001; Varga et al. 1996). Since 1996, when OTA was first detected in wine (Zimmerli and Dick, 1996), there have been numerous other reports confirming the presence of the mycotoxin on grape derived products (Bellì et al., 2002; Cabañes et al., 2002; Varga and Kozakiewicz, 2006; Visconti et al., 1999). At the present wine is considered, after cereals, as the second major source of OTA intake in Europe and strict EU legislation has been established, setting a maximum level for OTA of 2 μg $L^{-1}$ in wine musts and grape juice and of 10 μg $kg^{-1}$ for dried vine fruits (Commission Regulation No 123/2005 amending Regulation No 446/2001). This represents a matter of great concern for wine producers in Mediterranean countries in particular, since the highest levels of OTA have been recorded in wines which have been produced in this area. In the past decade studies have clearly indicated that fungi which are responsible for OTA accumulation in grapes belong to *Aspergillus* section *Nigri* (black aspergilli). Among these the main species appears to be *A. carbonarius* (Abarca et al., 2003; Battilani et al., 2003), which has also been found to be responsible for OTA accumulation in coffee and cocoa together with the well-

known *A. ochraceus*, several strains of which are now classified as *A. westerdijkiae*. (Frisvad et al., 2004; Taniwaki, 2006).

Structurally OTA consists of a polyketide which is believed to be derived from a dihydroisocoumarin group that is amide-linked to the amino acid L-phenylalanine. Its biosynthesis pathway has yet not been completely elucidated, although a number of putative pathways have been proposed (Harris and Mantle 2001; Huff and Hamilton 1979). Based on the molecular stucture of OTA it is clear that a number of enzymatic reactions are likely to be required for its biosynthesis: a polyketide synthase (PKS) for the synthesis of the polyketide dihydroisocoumarin, a cyclase, a chloroperoxidase or halogenase, an esterase and a peptide synthetase for ligation of the phenylalanine to the dihydroisocoumarin (Harris and Mantle 2001; Moss 1996, 1998). With respect to a polyketide synthase there is clear evidence of its involvement in OTA production in both *Aspergillus ochraceus* (O'Callaghan et al., 2003) and *Penicillium nordicum* (Karolewiez and Geisen, 2005). *Pks* gene expression has been shown to correlate with OTA production in *A. ochraceus*, where in addition two putative p450 monoxygenase genes, which are co-expressed together with the *pks* gene, also appear to be upregulated during OTA production, under different physiological condition, indicating their possible role in the biosynthesis (O'Callaghan et al., 2006b). In *Penicillium nordicum* other putative OTA biosynthetic genes have been reported including a non-ribosomal peptide synthetase (*otanps*PN) together with two genes which putatively encode a transporting protein (*otatra*PN*)* and a chloroperoxidase (*otachlPN*) respectively (Geisen et al., 2006). Expression of these genes appears to be coordinated and to be organised in a cluster, in a similar fashion to many other genes involved in the synthesis of secondary metabolites in fungi (Hoffmeister and Keller, 2007).

Recently other molecular studies have focused on the identification of genes involved in OTA biosynthesis in *A. carbonarius*, with particular attention on the identification of *pks* genes. In this respect Lebrihi and co-workers described the cloning of five different, highly diverse, ketosynthase (KS) domain sequences of putative polyketide synthase genes in *A. carbonarius* 2Mu134 (Atoui et

al., 2006). In this study we report on the use of degenerate primers targeting the KS domain which have allowed the identification of a 2.2 kb fragment of a new *pks* gene (*ACpks*), which appears to contain sequence corresponding to both the KS and acyltransferase (AT) regions, in an OTA producing strain of *A. carbonarius*. Furthermore we report that *ACpks* gene expression appears to correlate with OTA production in the fungus, under different physiological conditions, indicating a possible role for the product of this gene in OTA biosynthesis in *A. carbonarius*. Primers designed on the basis of the *ACpks* sequence were utilised in a preliminary screening to monitor the presence of the gene in the genomes of other *Aspergillus* isolates.

This work represents an important first step in increasing our understanding of the genetic mechanism of OTA biosynthesis in *A. carbonarius*, which is important considering its relevance as the main fungus responsible for OTA contamination in grapes and the pressing need to develop detection assays with high specificity to toxigenic properties.

## Materials and Methods

### Fungal strains and culture conditions

*Aspergillus carbonarius* ITEM 7444 (Agri-Food Toxigenic Fungi Culture Collection of the Institute of Sciences of Food Production, CNR, Bari, Italy), an ochratoxin A producer strain isolated from grapes, was used throughout this study. It was routinely grown at 25°C on potato dextrose agar (PDA) (Oxoid Ltd., Basingstoke, UK) for 5 days. Conidia (final concentration of $10^6$ spores/ml) were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of medium. Media used for this work were: Wickerham (40 g/l glucose, 5g/l peptone, 3 g/l yeast extract, 3 g/l malt extract); YES

(20 g/l yeast extract, 150 g/l sucrose); MM (6 g/l $NaNO_3$, 0.52 g/l KCl, 0.52 g/l $MgSO_4 \cdot 7H_2O$, 1.52 g/l $KH_2PO_4$, pH 6.5, 10 g/l glucose, 2 ml/l *Hutner's trace elements*); SGM (synthetic grape juice medium: 70 g/l glucose, 30 g/l fructose, 7 g/l tartaric ac., 10 g/l malic ac., 0.67 g/l $(NH_4)_2SO_4$, 0.67 g/l $(NH_4)_2HPO_4$, 1.5 g/l $KH_2PO_4$, 0.75 g/l $MgSO_4$ $7H_2O$, 0.75 g/l NaCl, 0.15 g/l $CaCl_2$, 0.0015 g/l $CuCl_2$, 0.021 g/l $FeSO_4$ $7H_2O$, 0.0075 g/l $ZnSO_4$, 0.05 g/l cathechin, pH 4.2). The SGM medium was modified by adding either $CH_3COOH$ or KOH to adjust the pH.

Incubation was carried out at 25°C shaking (150 rpm) (for Wickerham and YES media) or without shaking (for MM and SGM). The mycelium was harvested by filtration, frozen in liquid nitrogen and then stored at -80°C or lyophilized prior to nucleic acid extraction.

The filtered MM and YES liquid cultures and the lyophilized mycelium collected from SGM cultures were analysed for their ochratoxin A content.

Nine strains of *Aspergillus* spp. together with the *A. carbonarius* were used in a screening analysis for *ACpks*. All the strain have an ITEM accession number.

**Nucleic acid extraction and cDNA synthesis**

DNA was isolated using the Fungal DNA miniprep kit (E.Z.N.A.) (Omega Bio-Tek Inc., Doraville, GA) according to the manufacturer's protocol. Total RNA was extracted from frozen mycelium pulverised in liquid nitrogen using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA samples were treated with RNAse-free DnaseI (Promega, Madison, WI) to eliminate possible trace amounts of contaminating DNA. RNA aliquots were preserved at -80°C. First strand cDNA was synthesised using about 1.5 μg of total RNA, oligo $(dT)_{18}$ primer and the SuperScript III reverse transcriptase (Invitrogen, San Diego, CA) according to the manufacturer's protocol.

**PCR and RT-PCR, sequencing and data analysis**

PCR assays on *A. carbonarius* genomic DNA were performed using degenerate primers LC3for/LC5cREV (Bingle et al., 1999) at 1.2 μM and 0.8 μM respectively and AoLC35-12Lfor/AoLC35-12Rev (Dao et al., 2005) at 0.5 μM with an amplification protocol consisting of a denaturation step at 94°C for 5 min, 35 cycles of 30 s at 94°C, 50 s at 55°C and 1 min at 72°C, and an extension step of 5 min at 72°C.

Primers pairs PKac1for/rev, PKac2for/rev, PKac3for/rev, PKac4for/rev, designed in this study to target in a specific manner the four different fragments *Acpks1*, *ACpks Acpks3* and *Acpks4* respectively, were used at 0.3 and 0.5 μM for the amplification from genomic DNA and for first strand cDNA synthesis at the following conditions: 94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C for 50 s, 72°C for 50 s then a final extension step of 72°C for 5 min.

The primers CL1for/CL2rev (O'Donnell et al., 2000), were used to monitor calmodulin gene expression. These primers span two introns, which also allowed to check for DNA contamination.

For the analysis of *ACpks* expression at different pH values, the number of cycles used in the RT-PCR was varied to avoid amplification reaching a plateau and to allow a better normalization of template amounts. After different trials, PCR was set up at 23 cycles for calmodulin gene and 30 cycles for *ACpks* gene.

Two units of Taq DNA polymerase (Roche Applied Science, Indianapolis, IN) and dNTPs at 200 μM were used in each PCR experiment. Oligonucleotides were synthesised by MWG Biotech AG (Ebersberg, Germany), dissolved to 100 μM final concentration with sterile water and stored at -20°C. Sequences of primers designed for this study are listed in Table 1.

A negative control reaction (no DNA added) was included in all the PCR experiments.

The sequence of all cDNA and genomic amplicons were confirmed by sequencing analysis.

PCR products were sequenced directly or cloned into pGEM-T Easy with the pGEM-T Easy vector System kit (Promega) and then sequenced.

All sequence data were obtained using the ABI Prism Big Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystem, Foster City, CA). Reactions were analysed using a model 3100 Genetic Analyser (Applied Biosystem). Sequence similarity searches were performed using BLAST programs at NCBI (National Center for Biotechnology Information). Nucleotide and aminoacid alignments were performed with the Clustal W program.

**Identification of the partial sequence of *ACpks* gene**

The sequence of the *ACpks* gene was determined by genomic walking using the LA PCR *in vitro* cloning kit (TaKaRa BioMedicals, Shiga, Japan). Complete digestions of high molecular genomic DNA was performed with the enzymes *Eco*RI, *Hind*III, *Pst*I, *Sal*I and *Xba*I in the possibility that restriction sites could flank the region of interest. Adaptor cassettes, formed by double strand synthetic oligonucleotides with the restriction site at one end, were ligated to the genomic fragments for the creation of restriction libraries. A primary PCR was carried out for each of the libraries using adaptor and specific primers, these latter designed on the basis of the known fragment to walk upstream and downstream on the genome. The primary PCRs were followed by nested amplifications that utilised a 100 diluted primary PCR mixture as template to increase specificity with the use of nested primers, one targeting again the adaptor cassette and an inner primer specific for the genomic region of interest.

Cycling parameters for the primary touchdown PCR were, after denaturation at 94°C for 5 min, 5 cycles at 94°C for 20 s and a decreasing annealing temperature from 72 to 67°C for 3 min, followed by 32 cycles at 94°C for 20 s and 67°C for 3 min, and a final extension step at 67°C for 5 min. The conditions of the nested touchdown PCR were, after the denaturation step at 94°C for 5 min, 5 cycles at 94°C for 20 s and 72°C for 3 min, 20 cycles at 94°C for 20 s and 67°C for 3 min, followed by an extension step at 67°C for 5 min.

**Analysis of ochratoxin A in *A. carbonarius* cultures**

Acetonitrile and methanol (both HPLC grade) were purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Other reagents and standard of ochratoxin A (OTA) were purchased from Sigma–Aldrich (Milan, Italy). Filter papers (Whatman no. 4) were purchased from Whatman International Ltd., (Maidstone, UK). HPLC syringe filters (0.45 μm) were from Alltech (Deerfield, IL, USA). A stock solution of OTA was prepared in toluene:acetic acid (99:1 vol/vol) at a concentration of 1 mg/ml. Calibrant solutions for standard calibration curves were prepared by drying different aliquots of the ochratoxin A stock solution under a nitrogen stream that were successively reconstituted in the HPLC mobile phase (acetonitrile:water:acetic acid, 99:99:2 vol/vol/vol). Standard calibration curve was performed in the range 0.1-5.0 ng toxin injected (4 points).

Analysis of OTA in MM and YES liquid cultures of *A. carbonarius* ITEM 7444 was performed by directly injecting into the HPLC apparatus 100 μl of the liquid culture previously filtered through a 0.45 μm filter. For the analysis of OTA in fungal mycelium of *A. carbonarius* grown in SGM an extraction with a mixture of acetonitrile:water was necessary. In particular, the lyophilized mycelium was extracted with a mixture of acetonitrile:water (60:40 vol/vol) by shaking for 2 hr at room temperature. The ratio mycelium/extraction solvent mixture was 1g/12ml. After extraction the sample was filtered through a filter paper (Whatman N. 4) and the mycelium was discarded. An aliquot of the filtered extract (600 μl) was diluted with 400 μl of a mixture of methanol:water:acetic acid (70:130:5 vol/vol/vol) and vortexed for 30 sec. This solution was further diluted 10 times with HPLC mobile phase, filtered (0.45 μm) and 100 μl (equivalent to 0.5 mg of lyophilized mycelium) were injected into the HPLC apparatus.

The HPLC apparatus was a 1100 series LC system comprising a binary pump, an autosampler, a fluorescence detector (excitation wavelength 333 nm and emission wavelength 460 nm) from Agilent Technologies (Waldbronn, Germany). The column was a Waters Symmetry $C_{18}$ (150 mm × 4.6 mm, 5 μm particles) (Waters, Milford, MA, USA), preceded by a 0.5 μm Rheodyne guard filter.

The flow rate of the mobile phase was 1 ml/min. The detection limit was 0.4 ng/ml for liquid culture and 80 ng/g for dry mycelium. Confirmation of the identity of OTA by methyl ester formation was performed for selected positive samples by derivatizing the extracts with 14% $BF_3$-methanol as described elsewhere (Nesheim et al., 1992).

**Sequence accession number**

The nucleotide sequence of the *ACpks* gene of *A. carbonarius* has been deposited in DDBJ/EMBL/Gen Bank under accession number AM944567.

## Results

**Amplification and identification of KS domain sequences in *Aspergillus carbonarius***

Two pairs of degenerate primers namely LC3for/LC5cREV and AoLC35-12Lfor/AoLC35-12Rev were used with the aim of targeting KS domains from *pks* genes in the potent OTA producing strain *A. carbonarius* ITEM 7444. Using these primers four different fragments in the 300-600 bp range length were obtained. Following subsequent sequencing and comparative analyses three of these, namely *Acpks1*, *Acpks3* and *Acpks4*, were found to be identical to KS domain sequences already identified in *A. carbonarius* by Atoui et al. (2006). Specifically *Acpks1* (415 bp) displayed nucleotide identity (98%) to the Ac12RL3 clone, *Acpks3* (429 bp) to the AcKS10 clone (98%) and *Acpks4* (300 bp) to the AcKS9 clone (100%). The fourth KS domain sequence identified in this study, namely *ACpks*, was 618 bp long and exhibited the highest similarity (82%) to the coding

sequence of a predicted PKS protein of *A. niger*, resulted from sequencing and analysis of the CBS 513.88 strain genome. Moreover it showed an identity around 70% to a KS domain identified in *A. ochraceus* (clone AoKS9) by the same authors mentioned above. The nucleotide similarity among KS domains of *A. carbonarius* was in the range between 36 and 50%.

**_ACpks_ expression correlates with ochratoxin A production**

In order to examine a possible correlation between the expression of the four *pks* genes identified in this study and OTA production, *A. carbonarius* was grown in a variety of different media, which are known to affect the OTA biosynthesis. The transcription of each of the genes was monitored using a reverse transcription (RT)-PCR based approach, and OTA production was monitored in parallel by HPLC (Figure 1). When *A. carbonarius* was grown on synthetic grape medium (SGM), OTA production was initially observed on day 2 with levels increasing to reach a maximum level of 1.84 μg/g on day 4; with levels subsequently decreasing quite markedly thereafter to 0.66 μg/g on day 7 (Figure 1A). Analysis of *A. carbonarius* polyketide synthase gene transcript levels clearly indicate that the *ACpks* gene is expressed under these conditions, while no expression of the other three *pks* genes, namely *Acpks1*, *Acpks3* or *Acpks4* was observed (Figure 1B). The levels of *ACpks* gene transcripts seemed to correlate closely with OTA production.

Figure 2 represents the effects observed on OTA production and polyketide synthase gene expression when *A. carbonarius* was grown in MM (conducive) and YES (restrictive) media, both of which have previously been reported to affect OTA production in the fungus (Gallo et al., 2006). When *A. carbonarius* was grown on MM medium, low levels of OTA were produced following 3 days of growth, but a marked increase in OTA production was registered following 6 days of growth, while no OTA was detected when the fungus was cultured on YES medium (Figure 2A). The presence of *ACpks* transcript were observed in MM medium on days 3 and 6, with no

284 expression being observed in the fungus grown in YES, the restrictive medium, again suggesting a
285 link between *ACpks* gene expression and OTA production.
286 In contrast no clear differences were detected in the expression levels of *Acpks1*, *Acpks3* and
287 *Acpks4* under the two growth conditions tested (Figure 2B).
288 The similar expression levels of calmodulin gene in *A. carbonarius* grown on the SGM, MM and
289 YES media, showed that the changes observed with the *pks* genes were specific and not simply a
290 result of changes in overall gene transcription levels in the fungus.
291
292
293 **Expression of the AC*pks* gene during OTA biosynthesis at different pH**
294 The potential influence of culture pH on OTA production and *ACpks* expression was monitored
295 when *A. carbonarius* was grown in SGM medium at different pH values (Figure 3). Given that the
296 highest levels of OTA production had previously been observed at day 4 in SGM medium, this was
297 the time point at which OTA production was measured (Figure 3A), while gene transcript levels
298 were measured at days 3 and 4 (Figure 3B).
299 From Figure 3A it is clear that culture pH has a marked effect on OTA production in *A.*
300 *carbonarius*, with the highest levels of OTA amount produced (1.87 μg/g dry mycelium) at pH 4.2.
301 The production levels showed a 4-fold, a 12-fold and a 15-fold decrease as the pH of the growth
302 medium was increased to pH 6, pH 8 and pH 10 respectively. While slightly higher biomass yields
303 were observed in the pH 3-6 range than at the higher pH values. Values indicated represent OTA
304 production assessed per dry weight of mycelium at these different pH points. With respect to *ACpks*
305 transcript levels which were monitored on days 3 and 4 of fungal growth, these remained
306 unchanged at the different pH values. Again no difference in the expression levels of the
307 constitutively expressed calmodulin gene was observed when *A. carbonarius* was grown at these
308 different culture pH values.
309

**Cloning and characterisation of DNA regions flanking the *ACpks* gene fragment**

A 1500 bp genomic region adjacent to the 3' end of the initial *ACpks* gene fragment was subsequently cloned using a genome walking based approach. This resulted in the cloning of a *ACpks* gene fragment totalling 2188 bp, from the *A. carbonarius* genome, giving rise to a protein fragment of 729 aa (AM944567).

Both the acyltransferase (AT) and ketoacyl synthethase (KS) domains, characteristic of fungal PKS proteins, were identified in the *ACpks* sequence. BLAST comparisons confirmed the homology to fungal polyketide synthases with the best match (80% and 90% identity at the nucleotide and deduced amino acid levels respectively) to a hypothetical protein in *A. niger* which displays a strong similarity to the *Cochliobolus heterostrophus* polyketide synthase PKS1 (Kroken et al., 2003). Following a comparative analyses at the deduced aminoacid level with the *pks* genes which have so far been shown to be involved in OTA biosynthesis, *ACpks* displayed a 48% and 40% identity for the KS domain and a 35% and 26% identity for the AT domain to the *pks* of *A. ochraceus* and the *pks* of *P. nordicum,* respectively (Figure 4).

**Occurrence of the *ACpks* gene in black Aspergilli species**

To analyse the presence of the *ACpks* gene in species of *Aspergillus* Sect. *Nigri*, genomic DNA was isolated and subjected to PCR with the same specific primers (PKac2for/rev) and with the same amplification conditions used for *A. carbonarius*. The species considered showed different phylogenetic distances from *A. carbonarius* and included both producers and not producers of OTA. As illustrated in Figure 5 products were obtained in both *A. sclerotioniger* and *A. ibericus*, the most closely related species to *A. carbonarius* among the species tested, with the former known to be an OTA producer while the latter is not. From the sequencing analysis both these amplicons exhibited a nucleotide similarity of about 90% to the corresponding *ACpks* fragment and a 89% similarity between them. In *A. niger*, *A. lacticoffeatus* and *A. japonicus* bands of the expected length

were present, but they were too faint to be eluted and sequenced. In addition a second band is visible, suggesting a low specificity of the primers in these species. No products were obtained from either the *A. ochraceus* strain ITEM 7099, or from the recently described *A. uvarum* species, which has been isolated from grapes and which is known not to produce OTA (Perrone et al., 2008).

## Discussion

PKS proteins are commonly found in many fungi and are primarily involved in the synthesis of a wide variety of different secondary metabolites. The presence of multiple PKS genes in many fungal genomes is due to the ability of some genera of filamentous fungi to produce a high number of polyketide metabolites, of high chemical diversity and requiring more than one class of polyketide backbone. PKSs are multifunctional enzymes encoded by a single gene and typically possess up to eight types of functional domains (Bingle et al. 1999). The cloning and molecular characterisation of many of the genes encoding fungal PKSs has been greatly facilitated by the very conserved nature of these functional domains, which has allowed the design of gene probes and degenerate primers which have been used to isolate the gene fragments encoding the PKS domains (Cox et al., 2004; Nicholson et al., 2001). This approach has previously been successfully employed to characterise five KS domains belonging to putative PKSs in *A. carbonarius* (Atoui et al., 2006). While a probe designed on the basis of one of PKS sequences identified was subsequently used by the same authors to carry out a quantitative analysis by real-time PCR for the estimation of the fungus DNA presence in grape samples to correlate to OTA production (Atoui et al., 2007a). However a clear involvement of any of these PKS encoding DNA sequences in OTA biosynthesis in *A. carbonarius* has yet to be established.

362 It is clear that the genetic characterisation of the OTA biosynthetic genes in *A. carbonarius* is
363 necessary not only to allow the development of molecular diagnostic assays based on expression
364 profiling, but also to determine the molecular triggers which control OTA biosynthesis in the
365 fungus.
366 In order to achieve this we employed degenerate primers to amplify fragments of genomic DNA
367 encoding the KS domain of putative *pks* genes from a potent OTA producing strain of *A.*
368 *carbonarius*, which had been isolated from grapes. Using this approach we identified a novel *pks*
369 gene fragment, namely the *ACpks* gene, from this strain. RT-PCR was then employed to check
370 whether OTA production was preceded by *ACpks* gene expression. In the first instances we focused
371 on *A. carbonarius* cultures which had been grown on synthetic grape medium (SGM), a medium
372 that had previously been reported by the Lebrihi group to simulate the toxigenic potential of
373 *Aspergillus* spp. isolated from grapes This group has also previously reported that, unlike other
374 OTA-producing fungal species, the partitioning of OTA into the spores and mycelium is higher than
375 that secreted into the liquid medium in *A. carbonarius* (Atoui et al., 2007b). Thus it was necessary
376 to establish a method from the extraction of the mycotoxin from the mycelium, to allow an analyses
377 of the amount of OTA accumulated during fungal growth. We found that on SGM OTA production
378 reached a maximum by day 4 with a marked reduction from day 5 onwards up to day 7 (Figure 1A).
379 This decrease in OTA levels following maximum OTA production has been reported in other
380 ochratoxigenic *Aspergillus* and *Penicillium* spp. (Esteban et al., 2004; Geisen, 2004); and is
381 believed to be due to fungal degradation of the molecule (Abrunhosa et al., 2002; Varga et al.,
382 2000).
383 When transcription of the four putative *pks* genes, namely *Acpks1*, *Acpks3*, *Acpks4* and *ACpks* was
384 monitored with *A. carbonarius* being grown on SGM, only the transcriptional profile of the *ACpks*
385 gene appeared to mirror the observed trend in OTA production (Figure 1B). High levels of *ACpks*
386 transcription was observed on day 3, consistent with the maximum of OTA accumulation at day 4,
387 while little if any gene transcription was observed from *Acpks1*, *Acpks3*, *Acpks4*. This seems to

388 indicate a likely involvement for the product of this gene in OTA biosynthesis in *A. carbonarius*.
389 This hypothesis was further strengthened when it was observed that transcription of the *ACpks* gene
390 appeared to occur only in *A. carbonarius* cultures that were grown on OTA permissive (MM)
391 medium (Figure 2), with no detectable transcript levels being observed on the OTA restrictive
392 (YES) medium. In contrast no marked differences were observed in the transcript levels of the other
393 3 *pks* genes, under OTA permissive and restrictive conditions.
394 A number of different environmental factors are known to affect mycotoxin production in a variety
395 of different mycotoxigenic fungi including carbon and nitrogen sources, temperature, water activity
396 and pH (O'Brian et al., 2007; White et al., 2006). With respect to *A. carbonarius* there have been a
397 number of reports on the effects of different temperatures and $a_w$ values on OTA production (Bellì
398 et al., 2004; Bellì et al., 2005; Marin et al., 2006). From Figure 3 it is clear that culture pH affects
399 OTA synthesis. The analysis performed on mycelium grown in SGM at different pH values showed
400 a maximum levels of OTA production occurring at pH 4.2. This is similar to a previous report on
401 OTA production in *A. ochraceus*, where higher levels of OTA were produced in the lower pH range
402 (O'Callaghan et al., 2006b). Interestingly the expression levels of the *ACpks* gene as monitored by
403 RT-PCR on day 3 and 4 did not appear to change under the different pH conditions tested, despite
404 the observed differences in OTA production. This is in marked contrast to *A. ochraceus*, where the
405 reduced production of OTA at higher pH values was accompanied by a reduction in *pks* gene
406 transcript accumulation. The same was true for *otapksPN* expression in *Penicillium nordicum* where
407 expression was also down regulated under acidic conditions which accompanied the observed large
408 decrease in OTA biosynthesis (Geisen, 2004). One possible explanation for the constant levels of
409 *ACpks* expression, observed here, is that the polyketide formation step, catalysed by the product of
410 the *ACpks*, may not in fact be transcriptionally regulated at higher pH levels in *A. carbonarius* and
411 that the observed reductions in OTA levels may result from the down regulation of other genes
412 involved in OTA biosynthesis in this particular fungus. Other post-transcriptional regulatory
413 mechanisms could also be responsible, acting on protein stability and activity.

Sequence analysis of the 2188 bp fragment of the newly characterised *ACpks* gene indicates the presence of putative KS and AT domains. Comparative analyses shows a level of similarity at the deduced amino acid level, with the corresponding region of a putative *pks* protein determined from the annotated genome of the CBS 513.88 strain of *A. niger*. Interestingly *A. niger* is both closely related to *A. carbonarius* and, to a lesser extent, also responsible for OTA contamination in grapes (Cabañes et al., 2002; Chulze et al., 2006; Perrone et al., 2006). The *A. niger* protein has been identified as a hybrid NRPS/PKS, with a strong similarity to the PKS1 of *Cochliobolus heterostrophus* (Yang et al., 1996). Such hybrid NRPS/PKS clusters, containing domains characteristic of both NRPSs and PKSs, have been found in many microbial genomes to be involved in the synthesis of secondary metabolites. However in the *A. niger* genome a different PKS, belonging to a putative ochratoxin cluster identified on the basis of its similarity to OTA PKS fragment of *A. ochraceus*, has been reported (Pel et al., 2007). Although the diversity of PKSs in fungi has been extensively investigated using comparative and phylogenetic methods (Nicholson et al., 2001; Varga et al., 2003), O'Callaghan and Dobson (2006a) claimed that phylogenetic relationship analysis based on a single domain or portion of PKS sequence may not be sufficient to predict the potential gene function, considering the complex structures of these proteins. As evidence of this, the *otapksPN* gene from *P. nordicum* was noted to have much less homology with the *otapks* of *A. ochraceus* compared to other fungal *pks* genes (Karolewiez and Geisen, 2005), and the OTA pathway genes were found to be similar in the producing species *P. nordicum* and *P. verrucosum*, except for the polyketide synthase gene (Geisen et al., 2006). The *ACpks* gene which we are proposing here to be involved in OTA biosynthesis in *A. carbonarius* displays a similar degree of sequence identity at the KS and AT domains to the OTA *pks* genes of *A. ochraceus*, which belongs to a different section of the same genus, and of *P. nordicum*, which is more distant phylogenetically. These results confirm the fact that the ochratoxin polyketide synthase genes in different ochratoxigenic fungi are more diverse than expected.

Black *Aspergilli* are considered as major sources of ochratoxin contamination in several important agricultural commodities and in particular they are responsible for OTA accumulation in grapes. As they represent one of the most problematic groups from an identification standpoint, much interest has recently been focused on taxonomic studies to address the identification of OTA producing species within the section *Nigri* (Abarca et al., 2004). In this respect *A. sclerotioniger* and *A. ibericus* have recently been identified as the species phylogenetically most related to *A. carbonarius*. *A. sclerotioniger*, isolated from green coffee beans, was described as an effective OTA producer (Samson et al. 2004), while no OTA production was documented for *A. ibericus*, a new species isolated from grapes (Serra et al*.,* 2006). Using PCR primers designed on the basis of the *A. carbonarius* gene, putative *pks* homologues, within the KS domain appear to be present in the genome of both these fungal species (Figure 5). While this may demonstrate the potential capability to produce OTA, expression of these genes is likely to be dependent on different factors. For example, other than the biotic and abiotic conditions affecting the effective synthesis of mycotoxin, it should also be considered that the presence and integrity of the remaining genes of the OTA biosynthetic pathway, which are as yet unidentified in the genus *Aspergillus*, will also need to be present.

Since not all *Aspergillus* species can produce OTA, it is interesting to speculate that this biosynthetic ability may in fact have been lost or gained a number of times during the evolution of the genus. Thus the further identification and molecular characterisation of genes involved in OTA synthesis may aid in our overall understanding of the evolutionary process of mycotoxin production. An enhanced knowledge of OTA biosynthetic genes is also clearly a key in the development of experimental systems to study the environmental and nutritional influences on OTA production, as well as for the generation of improved specific detection tools which are required to help monitor and quantify OTA levels in food products.

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682 **Fig.1 (A)** Ochratoxin A production by *A. carbonarius* ITEM 7444 on synthetic grape juice medium
683 (SGM). OTA accumulation at different incubation times was determined by HPLC. Error
684 bars denote the standard error of the mean of two replicates from independent cultures. **(B)**
685 RT-PCR analysis of *pks* genes and of calmodulin gene in *A. carbonarius* ITEM 7444
686 producing OTA in SGM. Genomic DNA of *A. carbonarius* was used as positive control.
687
688
689 **Fig.2 (A)** Ochratoxin A production by *A. carbonarius* ITEM 7444 on MM and YES media
690 (supporting and non supporting OTA production, respectively) after 3 and 6 days of
691 incubation. Data are the means of two independent cultures. **(B)** RT-PCR analysis of *pks*
692 genes and of calmodulin gene in *A. carbonarius* ITEM 7444, performed on RNA of 3-day-
693 old and 6-day-old mycelium grown in MM and YES media. Genomic DNA of *A.*
694 *carbonarius* was used as positive control.
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699 **Fig.3 (A)** OTA accumulation in *A. carbonarius* after 4 days growth on SGM at different pH values.
700 Curve represents fungal biomass. Error bars denote the standard error of the mean of two
701 replicates from independent cultures. **(B)** RT-PCR analysis of *ACpks* gene and of calmodulin
702 gene in *A. carbonarius* ITEM 7444, grown in SGM medium at different pH values RT-PCR
703 here represented was performed on RNA from 3-day old mycelium. Genomic DNA of *A.*
704 *carbonarius* was used as positive control.
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**Fig.4** Alignment of the deduced amino acid sequences of KS and AT partial domains of *A. carbonarius ACpks* with the corresponding regions of *P. nordicum* (AY196315) and of *A. ochraceus* (AT domain from AY272043; KS domain is under submission).

**Fig.5** PCR products obtained from genomic DNA of various Aspergilli isolates using *ACpks* specific primers. laneM:1 kb ladder marker. The isolates used are reported as OTA producers (Ac: *A. carbonarius*, As: *A. sclerotioniger* ITEM 7560; At: *A. tubingensis* ITEM 4496; An: *A. niger* ITEM 7096; Al: *A. lacticoffeatus* ITEM 7557; Ao: *A. ochraceus* ITEM 7099) and not OTA producer (Ai: *A. ibericus* ITEM 6602; Aj: *A. japonicus* ITEM 7034; Aa. *A. aculeatus* ITEM 7046; Au: *A. uvarum* ITEM 4843)

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**Table.1** Primers designed in this study

| Primer code | Primer sequence (5'→ 3') |
|---|---|
| PKac1for | TCG AAG TAC TGG CAG CGA TC 3' |
| PKac1rev | CTT CCT TTG TCC TCG ACA |
| PKac2for | TGA TCC TCG AGG TGG TGT A |
| PKac2rev | TTC GAC GTA GGT GGT ATC GA |
| PKac3for | CCA GTG TGA CCG TTG ATA C |
| PKac3rev | AAG TCG GTA TCT GCT GGA |
| PKac4for | TAA TAG CCG CCA GCA ACC TGA |
| PKac4rev | AAC TGA CAC CGG TCT TCA G |
| AC2GW5int | ATT CGT AAA TGA CCC GCA CAG GAC GGA |
| AC2GW5ext | CGG TGA CTG TGT AGC TGG GGT AAT ATT C |
| AC2GW3ext | AGT GGC GAC TGT GAC ACA TCC ATC ATT G |
| AC2GW3int | ACA TCT TCA TCA CCA TGA CCG ACC TGG |
| AC2-3IIGWext | GTC AGC ATC AAC TCG TTT GGC TAC GGA |
| AC2-3IIGWint | GTG CCA CTA ACC ACG CAT TCC GAC AA |

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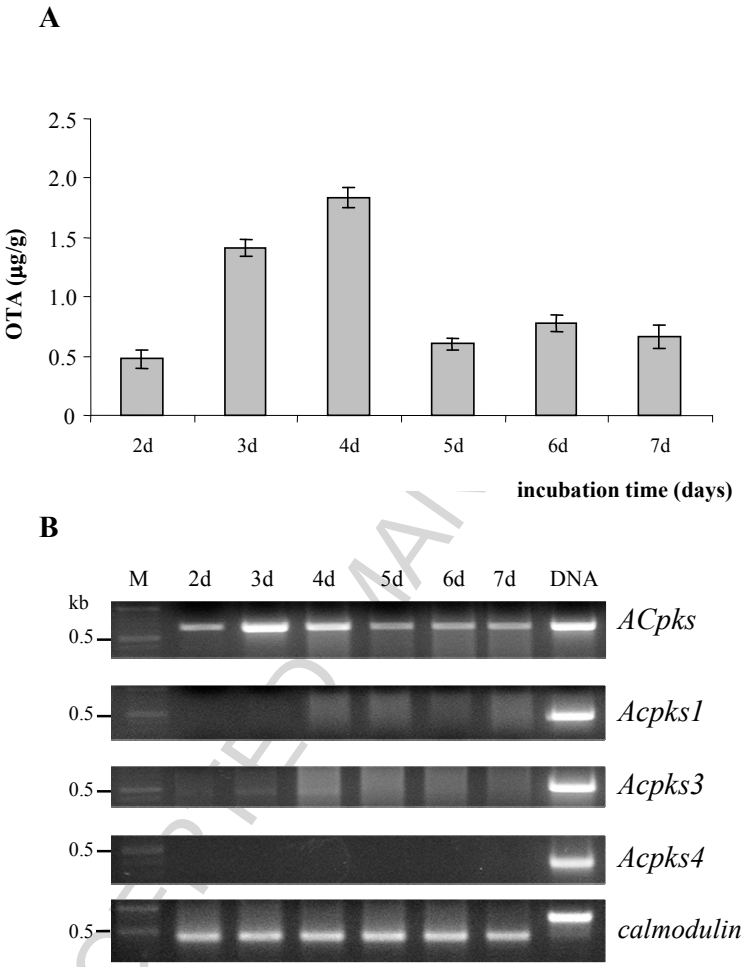
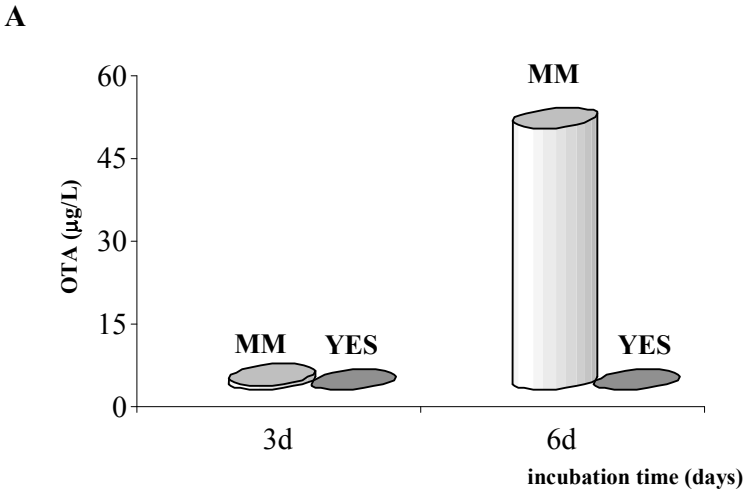
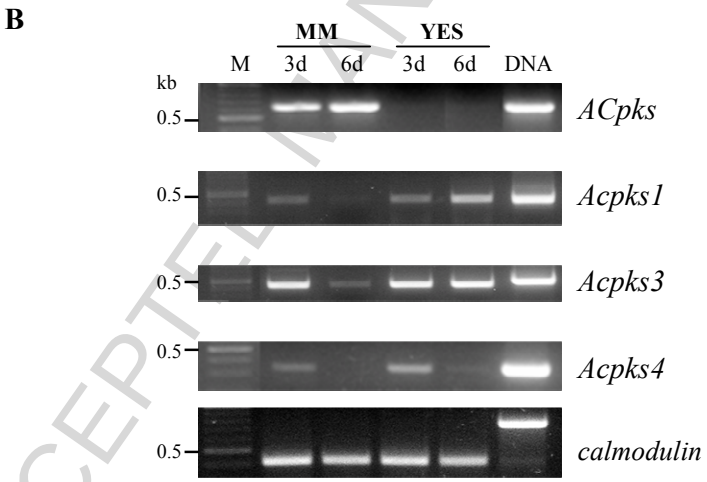
**Figure 1**




Gallo A., Perrone G., Solfrizzo M., Epifani F., Abbas A., Dobson A. D.W. and Mulè G.

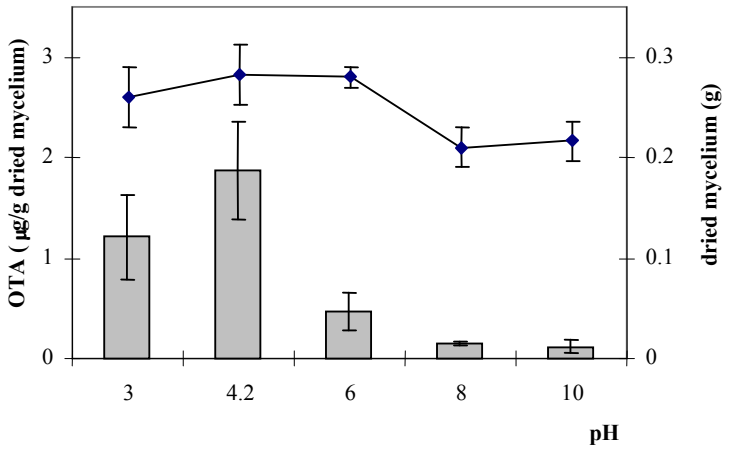


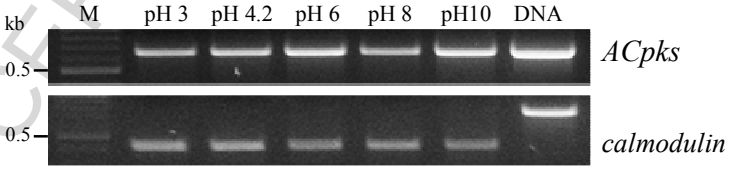

Gallo A., Perrone G., Solfrizzo M., Epifani F., Abbas A., Dobson A. D.W. and Mulè G.

**Figure 3**

**A**




**B**



**ketoacyl synthetase domain**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780
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782 **acyltransferase domain**
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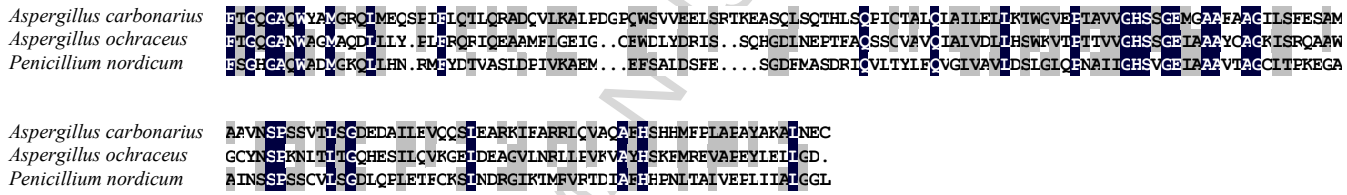


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785 **Figure 5**
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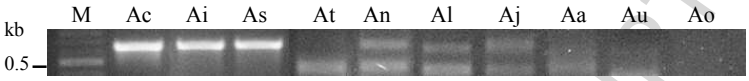




Gallo A., Perrone G., Solfrizzo M., Epifani F., Abbas A., Dobson A. D.W. and