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GENETIC APPROACH TO TARGET PUTATIVE PKS GENES IN ASPERGILLUS SECTION NIGRI SPECIES PRODUCING OCHRATOXIN A

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Abstract: A 700 pb PCR derived DNA fragment was isolated from *Aspergillus carbonarius*, *Aspergillus niger* and *Aspergillus tubingensis* using degenerated primers (LC1-LC2c) and two new designed primer pairs (KSLB-LC6) for *Aspergillus niger* and (AFl1F-LC2) for *Apergillus tubingensis* developed for the acyl transferase (AT) and the KS sequences of fungal PKSs genes. DNA from the most of black *Aspergillus* species currently recognized was tested. Herein, we report on the identification and characterization of a part of novel putative OTA-polyketide synthase gene in *A. carbonarius* "AC Pks", *A. niger* "AN Pks" and *A. tubingenis* "AT Pks". The sequences were aligned and analyzed using phylogenetic methods. Primers used in this study showed general applicability and other *Aspergillus* species belonging to section Nigri were successfully amplified specially in *A. niger* and *A. tubingenis*. The predicted amino acid sequences "AC Pks" displayed 66 to 81% similarities to different polyketide synthase genes while "AN Pks" similarities varied from 68 to 71% and "AT Pks" were from 81 to 97%. The AT and the KS sequences appeared to be specific for a particular type of fungal PKSs and were related to PKSs involved in different mycotoxin biosynthesis pathways, including ochratoxin A. The sequences presented in this work have a high utility for the discovery of novel fungal PKS gene clusters.

Keywords: PKS genes; OTA biosynthesis; *Aspergillus* Nigri; sequence analysis

Abreviations: Pks (Polyketides Syntase; AC Pks (*Aspergillus carbonarius* Polyketides Syntase) ; AN Pks (*Aspergillus niger* Polyketides Syntase) AT Pks (*Aspergillus tubingesis* Polyketides Syntase)

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1. Introduction

Fungal polyketide synthases (PKSs) are responsible for the biosynthesis of several mycotoxins and other secondary metabolites. Ochratoxin A (OTA) is one of fungal secondary metabolite [1-8-22-33]. It is a potent teratogen, cancerogen for humans [17-21-27]. OTA is a polyketide derived from the sequential condensation of multiple acetate units by a polyketide synthase (PKS). This mycotoxin is derived from the fungal polyketide biosynthesis pathway, which is partly elucidated [11]. The contribution of the species included in the *Aspergillus* section Nigri group, mainly *Aspergillus carbonarius* and the members of the *A. niger* aggregate (*A. niger* and *A. tubingensis*) in the contamination of grapes by ochratoxin A (OTA) has been recently reported worldwide [16-23]. In fungi, genes involved in the biosynthesis of secondary metabolites are often clustered, and clusters may include one or more regulatory genes [6-28].

The most clusters associated with the polyketide biosynthesis contain a single PKS gene and several genes encoding enzymes [2-12-14].

The ochratoxin biosynthesis involves two fatty acid synthases, which produce a fully reduced hexanoyl chain that acts as a primer for the PKS to complete the backbone [10]. In spite of a remarkable variety of end products, the individual polyketide biosynthetic pathways apparently follow a common basic reaction scheme. The key chain-building step of this reaction scheme is a decarboxylative condensation analogous to the chain elongation step of classical fatty acid biosynthesis [5-13]. In the biosynthesis of most polyketide metabolites, a group of multifunction enzyme system called polyketide synthases (PKSs) catalyzes the successive condensation step of small carbon precursor acid [24]. A typical fungal PKS is composed of principal domains including ketosynthetase (KS), acyltransferase (AT) and acyl carrier protein (ACP) and optional domains including dehydratase (DH), enoyl reductase (ER), ketoreductase (KR) and thioesterase (TE) [9]. In this study, we report the characterization of PKSs genes (AC Pks) required for the biosynthesis of OTA in *A. carbonarius* using degenerate primers (LC1-LC2c) and two designed primer pairs (KSLB-LC6) and (AFL1F-LC2c) to target respectively, PKS genes in *A. niger* and *A. tubingenis* as OTA producing fungi in grapes.

2. Materiel and Methods

2.1. Fungal strains and culture condition

The isolated fungal cultures for *A. carbonarius*, *A. niger* and *A. tubingensis* were grown on MEA (Malt Extract Agar–DIFCO) supplemented with 100µg/ml chloramphenicol (Sigma) for 7 days for sporulation. Cultures were incubated at 25°C prior to DNA extraction. Chemicals, unless specifically referenced in the text, were purchased from Difco Laboratories (Detroit, Mich.).

2.2. Fungal DNA extraction

Mycelia were harvested at the appropriate time, weighted, and frozen immediately at -80°C. DNA was isolated using the Fungal DNA miniprep kit (QIAGEN-France) according to the manufacturer's protocol. DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA).

2.3. Amplification of fungal DNA

PCR assays on *A. carbonarius* genomic isolated DNA froma all samples were performed using degenerate primers (LC1-LC2c) [26]. The PCR was performed as follows: "36 sequential cycles carried out after the initial denaturising cycle (95°C for 8min). Each cycle consisted of three steps (95°C for 1min, 55°C for 1min and 72°C for 3min) followed by a final extension step of 72°C for 10 min".

Specific primer sets were designed based on sequence alignments of the PKS gene of more than fifty *Aspergillus* and *Penicillium* strains from different origins, and retrieved from databases. All sequences were edited and aligned by Clustal W method using the MegAlign program of DNA STAR software (Lasergene, Wisconsin, USA).

The both new pair primers, (KSLB/Lc6) and (Afl1F/LC2c), were designed in the laboratory (Table 1).

Table 1. PCR primers used to identify PKSs genes in *Aspergillus* strains producing OTA.

Primer code	Primer sequence (5'-3')	Annealing temperature (°C)
LC1 LC2c	GAYCCNMGNTTYTTYAAYATG GTNCCNGTNCCRTGCATYTC	55
KSLB LC6	ATGACIATHGAYACIGCITG CCRTGIGCYTCRAARAAYTG	55
Afl1F LC2R	GARGCICCICARATGGAYCC GTNCCNGTNCCRTGCATYTC	55
PU PR	CGTTGTAAAACGACGGCCAGT GTACCAGTATCGACAAAGGAG	52

2.4. DNA Cloning and Sequencing

The purified PCR products were ligated into the *pGEMT* vector (Promega), as instructed by the manufacturer. This vector was used to transform into competent cells of *Escherichia coli* (JM 109) using the primers PU-PR as insert-specific primers in the vector *pGEMT*. Transformed colonies of *E.Coli* JM 109 were selected on Luria-Bertani (LB) plates containing ampicillin, isopropythio-β-D-galactoside (IPTG) and 5- bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Sambrook et al., 1989). Individual clones were used to inoculate 5ml of LB broth incubated and were cultivated overnight at 37°C. Plasmid DNA was isolated from *E. coli* on a small scale preparation (miniprep) to identify the recombinant colonies.

The ABI Prism "CEQ™ 8000 Genetic Analysis System" was used for PCR sequence analysis. *Aspergillus* PKS sequences obtained were compared against other sequences available on-line with the Basic Local Alignment Search Tool algorithm (Blast, National Center for Biotechnology Information, National Institutes of Health).

3. Results

We looked at Pks sequencing data from recognized fungal strains that produce OTA, including A. carbonarius, A. niger, and, most recently, A. tubingenis, which has been identified as an OTA-producing species. A. carbonarius is the principal producer of OTA in grapes and its derivatives.

3.1. Amplification and identification of Pks gene sequence in A. carbonarius

One pair of degenerate primers namely LC1/LC2c was used with the aim of targeting KS domains from Pks genes in the potent OTA producing strain *A. carbonarius, A. niger and A. tubingenis* isolated from Tunisian grapes. The result was a fragment of 700 bp. Following subsequent sequencing and comparative analyses of this sequence, namely AC Pks was found to be identical to KS domain sequences already identified in *A. carbonarius* by Atoui et al. (2006). Specifically, AC Pks (Figure 1) exhibited the highest similarity (81%) to the coding sequence of a predicted PKS protein in *A. oryzae*, resulted from sequencing and analysis of the AP007162 strain. Moreover, a similarity, around 76%, to KS domains was obtained with *A. ochraceus* (AAS98198) and *A. terreus* (CAB44699). While a similarity of 66% was obtained with *A. flavus* (AAS90022), (See Table 2).

Table 2. Similarities between sequences encoding proteins putatively involved in the synthesis of ochratoxin A precursors isolated from *A. carbonarius* species and PKSs reported in several toxigenic fungi.

Fungal PKSs	*A. oryzae* (AP007162)	*A. ochraceus* (AAS98198)	*A. terreus* (CAB44699)	*A. flavus* AAS90022)
Similarities (%)	81	76	76	66

The deduced PKS sequence "AC PKS" was aligned with PKSs submitted online (Figure 1)

Figure 1. Alignment of the deduced amino acid sequences "AC Pks" of *A. carbonarius* (Tun) isolated from Tunisian grapes with the corresponding PKSs regions *of A. oryzae* (Accession No. AP007162), *A. ochraceus* (Accession No. AY272043), *A. terreus* (Accession No. CAB44699), and *A. flavus* (Accession No. AAS90022).

The isolated Pks sequence "AC Pks" obtained is described as following (Figure 2).

Figure 2. Pks gene sequence from *A. carbonarius.*

3.2. *Amplification and identification of PKS gene sequences in A. niger and A. tubingensis*

To analyze the presence of the AN Pks gene *in A. niger* and AT PKS *in A. tubingensis*, genomic DNA was isolated and subjected to PCR with the same specific pair primer (LC1/LC2c) and with the same amplification conditions used for *A. carbonarius*.

The two species (*A.niger* and *A. tubingensis*) showed positive response with these degenerate pair primers and a full similarity was obtained with the aminoacid sequence

"AC PKS". In order to isolate new PKSs genes from *A. niger,* and *A. tubingensis* a new molecular approch was optimized by using new primers designed in the laboratory (Table 1).

3.2.1. Cloning of ANPks gene in A. niger

After PCR reaction, a 700 bp fragment was amplified using the pair primer (KSLB–LC6). The PCR products were cloned into *pGEMT* plasmid and sequenced. Alignment of the sequenced fragments was performed to search for consensus. From the sequencing analysis, the amplicons exhibited a similarity of about 71% to the corresponding PKS of *P. chrysogenum* (CAP95405) and *A. ochraceus* (AAS98204). AN PKS gene sequence revealed a similarity of 68% with *A. carbonarius* (ACH47947) and *A. oryzae* (BAE56814) (see Table 3).

Table 3. Comparison of the deduced amino acid sequence for *Aspergillus niger* using (KSLB-Lc6) and fungal PKSs sequences submitted in Genbank.

Fungal PKSs	*P. chrysogenum* (CAP95405)	*A. ochraceus* (AAS98204)	*A. carbonarius* (CAB44699)	*A. oryzae* (BAE56814)
Similarities (%)	71	71	68	68

The new deduced protein sequence was aligned using genetic tools (Figure 3**).**

Figure 3. Alignment of the deduced amino acid sequences "*AN PKS*" of *A. niger* (Tun) isolated from Tunisian grapes with the corresponding PKS regions of *A. carbonarius* (Accession No. ACH47947), *A. ochraceus* (Accession No. AAS98204), *A. oryzae* (Accession No. BAE56814), and *P. chrysogenum* (Accession No. CAP95405).

```
Consensus        HQGVGALRSGDCHLAVIAASNLI--FSPREYIAASKMHLLSPTGRCRMWDENADGYARGE
A.niger(Tun)     HQGIQSLRTNESQTACIAGANLMETLSPELFLTESALHL-SPEGKCH-WDDRADGYARGE
A.carbonarius    HQG+ +LR+ +    A IA +NL+    SP   ++   S +HL SP G+C   WD+ ADGYARGE
A.ochraceus      HQ IQ+LR+ ES+ A   AG+NL+   L PE ++ ES L   LSP+G+     WD RA+GYA G+
A.oryzae         HQ + SL+   E++ + +AGANL+   L P +++ ES LH LSPE +     WD   A+GYARGE
P.chrysogenum    HQ +QSLRT E+   + + GANL   TL+P++F      SA   LS +GK + +D RA GY RGE

Consensus        GIAAVVLKRLGEAIADGDPIESVIRATGVNADGRSMGITMPSSMAQSQLIRSTYASIGLS
A.niger(Tun)     GIVVVFLKRLSDALADGDHIEAIIRDSGVNQDGRTRGIT-PSSEAQSALIEKVYRRSGLD
A.carbonarius    GI   V LKRL +A+ADGD IE++IR +GVN DGR+ GIT PSS AQS LI    Y    GL
A.ochraceus      G+   + LK+LS AL DGD IE +IR +GVNQDGRT+GIT PSS+AQ+ LI + Y+   GLD
A.oryzae         G    V+LK LS AL DGD IE IIR +GVN DGRT+GIT PSS AQ+ LI    YRR+GLD
P.chrysogenum    G+   + +KRLSDALA GD I A+IR+S +NQDG+T   IT PS EAQ AL+    Y+++GLD

Consensus        PIVRPEDRCQFFEAHG
A.niger(Tun)     PTA-PWGRCQFFEAHG
A.carbonarius    P   P  RCQFFEAHG
A.ochraceus             R QFFEAHG
A.oryzae         P+      R QF E HG
P.chrysogenum    P         Q+FEAHG
```

Here is a description of the *A. niger* PKS sequence that was determined (Figure 4).

Met Met

I Q A D P A Q R L A L L T A Y E A L E A G F I P D S S P S T Q R D R V G I F Y G T S D D Y R E I N S

Met

G Q D I D T Y F I P G G N R A F T P G R I N Y Y F K F S G P S V S V D T A C S S S L A A I H A C N S

I W R N D C D A A I A G G V N I L T N P D N H A G L D R G H F L S T T G N C N T F D D G A D G Y

C R A D G I G S I V L K R L E D A E A D

Figure 4. PKS gene sequence from A. niger.

Fungal PKSs	*A. terreus* (BAB88689)	*A. flavus* (AAS89999)	*A. ochraceoroseus* (ACH72912)
Similarities (%)	87	82	81

3.2.2. Cloning of ATPks gene in A. tubingenis

PCR primers (AFL1F-LC2c) designed in the laboratory were used to target Pks genes in *A. tubingenis* species. Products obtained from the PCR reactions were sequenced and shown to be fragments from as-yet undiscovered PKS gene. The sequence fragments displayed similarities with *P. thomii* (97%), *A. niger* (94%), *A. ochraceus* (88%), *A. terreus* (87%), *A. flavus* (82%) and 81% with *A. ochraceoroseus* (Table 4).

Table 4. Similarities of sequences encoding pks putative proteins isolated from Aspergillus tubingensis and fungal PKSs from toxigenic strains.

Fungal PKSs	Ketosynthase of *P. thomii* (AAS60178)	*A. niger* (CAK38306)	*A. ochraceus* *(AAS98198)*
Similarities (%)	97	94	88

The alignment of the predicted AT PKS was done (Figure 5).

```
Consensus            IQVDPAQRIALMTAYEAIEQAGIVPDATPSTRKDRVGVFYGVTSNDWMETNSAQNIDTYFI
A.tubigensis(Tun)    IQADPAQRLALLTAYEALE-AGFIPDSSPSTQRDRVGIFYG-TSDDYREINSGQDIDTYFI
A.ochraceus          IQ DPAQR+AL+TAYEA+E AG +PD++PST++DRVG+FYG TS+D+ E NS Q+IDTYFI
A.terreus            I DPAQRLA+ T YEA+E AG + + +PSTQ+DRVG+F+GT SDD+R +NSGQD+ TYFI
P. thomii            IQADPAQRLALLTAYEALE AGFIPDS+PSTQ++RVG+FYG TSDDYREINSGQDIDTYFI
A.niger              IQADPAQRLA+ TAYEA+E AG +P+++PSTQ DR+G+FYG TSDD+RE+NSGQ++DTYFI
A.flavus             IQ DPAQR+AL++ YEA+E AG +PD++PSTQRDR+G+F+G TS+D+ E N+ Q+IDTYFI
A.ochraceoroseus     IQ DPAQR+AL++ YEA+E  G +PD++ STQR+R+G+F+G TS+D+ E N+ Q+IDTYFI

Consensus            PGGNRAFIPGRLNYFFKFSGPSYSVDTACSSSLASLHVACNALWRGDIDTAIAGGTNVLT
A.tubigensis(Tun)    PGGNRAFTPGRINYYFKFSGPSVSVDTACSSSLAAIH-ACNSIWRNDCDAAIAGGVNILT
A.ochraceus          PGGNRAF PGR+NY+FKFSGPS SVDTACSSSLA++H ACN++WR D D AIAGG N+LT
A.terreus            PGGNRAF PGRI+Y+F+FSGPS+S+DTACSSS AAI A C+ +WR +CD AIAGG NILT
P.thomii             PGGNRAFTPGRINYYFKFSGPSVSVDTACSSSLAAIH ACNS+WRN+CD+A+AGGVNILT
A.niger              PGGNRAF PGRI+Y+F+F GPS+S+DTACSSS AAI  AC  +WR +CD A+AGGVN+LT
A.flavus              GGNR F PGRIN+ F+F+GPS + DTACSSSLAAIH ACNS+WR DCD A+AGG N++
A.ochraceoroseus     PGGNR F PGRIN+ F+FSGPS S DTACSSSLAAIH ACNS+WR DCD A+AGG N++

Consensus            NPDFTAGLDRGHFLSRTGNCKTFDDGADGYCRGEGVGTVILKRLEDAVAD
A.tubigensis(Tun)    NPDNHAGLDRGHFLSTTGNCNTFDDGADGYCRADGIGSIVLKRLEDAEAD
A.ochraceus          NPD  AGLDRGHFLS TGNC TFDDGADGYCR +G+G+++LKRLEDA AD
A.terreus            NP DNAGLDRGHFLSTTGNCNTFDD A GYCR+D +GS++LKRLEDAEAD
P.thomii             NPDNHAGLDRGHFLS TGNCNTFDDGADGYCRGEGVGTVILKRLEDAVAD
A.niger              N PDN  GLDRGHFL+ GNCN FDD ADGYCR+D +G+++LKRLEDA  D
A.flavus              PD H GLD+G FLS TGNC  +DD ADGYCRA+G+G++ +KRLEDA AD
A.ochraceoroseus      PD H GLD+G FLS TGNC  FDD ADGYCRA+G+G++ +KRLEDA AD
```

Figure 5. Alignment of the deduced amino acid sequences "AT PKS" of *A. tubingensis* (Tun) isolated from Tunisian grapes with the corresponding PKS regions of *P. thomii* (Accession No. AAS60178), *A. niger* (Accession No. CAK38306), *A. ochraceus* (Accession No. AAS98198), *A. terreus* (Accession No. BAB88689), *A. flavus* (Accession No. AAS89999) and *A. ochraceoroseus* (Accession No. ACH72912).

The derived Pks sequence from *A. tubingenis* is characterized as follows.

```
                  Met                       Met
                   ↓                         ↓
IQADPAQRLALLTAYEALEAGFIPDSSPSTQRDRVGIFYGTSDDYREINS
                                                   Met
                                               ↓
GQDIDTYFIPGGNRAFTPGRINYYFKFSGPSVSVDTACSSSLAAIHACNS
IWRNDCDAAIAGGVNILTNPDNHAGLDRGHFLSTTGNCNTFDDGADGY
CRADGIGSIVLKRLEDAEAD
```

Figure 6. Pks gene sequence from *A. tubingenis*

4. 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 [4-18-25].

In the present work, degenerate PCR primers (LC1-LC2c) were designed matching regions of specific homology and the primers were used in PCR reactions with fungal genomic DNA from a number of known polyketide producing species. Additionally examined for *A. niger* and *A. tubingensis*, these primer pairs displayed complete resemblance to *A. carbonarius*. This method has been used before with success to characterize five KS domains that are putative PKSs in A. carbonarius. [3]. However, a clear involvement of any of these PKS encoding DNA sequences in OTA biosynthesis in *A. carbonarius* has yet to be established. Furthermore, we report that primers designed based on the AC Pks sequence were utilized in a preliminary screening to monitor the presence of the gene in the genomes of *Aspergillus* isolates (*A. niger* and *A. tubingenis*). Degenerate PCR primers (LC1 and LC2c) were also used for the amplification of ketosynthase domain fragments from fungal PKS genes to characterize endophytes fungi [32].

Amino acid sequences revealed by utilizing these primers for Aspergillus section Nigri revealed similarities between PKSs genes for fungal toxigenic strains that ranged from 68% to 77%. Using degenerate primers that target the KS domain, a new PKS gene (AC PKS) was discovered in this study, and it appears that the sequence in this gene corresponds to both PKSs in an OTA-producing strain of *A. niger* and *A. tubingenis*. Here, the new molecular approach used in this study offers new possibilities to study PKSs genes in both species *A. niger* and *A. tubingenis* by using (KSLB-LC6) and (Afl1F-LC2c), respectively. Products obtained from these reactions were sequenced and shown to be fragments from as-yet undiscovered Pks gene clusters. The fragments could be used in blotting experiments with either homologous or heterologous fungal genomic DNA. These PKSs genes displayed a high degree of similarity with other PKSs genes encoding enzymes involved in the biosynthesis of polyketide secondary metabolites. Here, we focused on three putative PKSs genes [33].

According to other studies, the highly conserved nature of these functional domains greatly facilitated the cloning and molecular characterization of many genes encoding fungal PKSs. This allowed the design of gene probes and the use of degenerate primers to isolate the gene fragments encoding PKS sequences [7-25].

In addition, 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 his co-workers

described the cloning of five different, highly diverse, ketosynthase (KS) domain sequences of putative polyketide synthase genes in *A. carbonarius* [3-34].

In line with other research projects [9-31], until now fewer information about screening of PKSs in *A. niger* and *A. tubingenis* and this work represents an important 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 followed by *A. niger* and *A. tubingenis.*

However, Martnez-Culebras and his collators [19] have also created primers suitable for amplifying AT domain sequences in strains belonging to the *A. niger* aggregate and primers showed general applicability for other *Aspergillus* species belonging to section Nigri. These developments address a pressing need for the development of detection assays methods with high specificity to toxigenic properties. The molecular approach used to target PKSs genes in *A. niger* and *A. tubingenis* using primers (KSLB-LC6; AFL1F-LC2c) may be a useful molecular tools to facilitate the cloning of novel fungal polyketide synthase genes. Moreover, products obtained from these PCR reactions were sequenced and the deduced proteins shown to be fragments from as-yet undiscovered PKSs genes. These amino acid sequences displayed similarities (68%-97%) with PKSs genes involved in the biosynthesis pathway of several mycotoxins such as aflatoxins [20-29].

The major objective of this research is to identify PKSs by cloning the genes implicated in the production of OTA in fungi. Finding the genes that control the manufacture of these substances and completely understanding how the genes control how the compounds cluster together from the fundamental components found in fungal cells will be the ultimate goals. Additionally, we are interested in carrying out rational genetic engineering studies to change the genes and produce new materials that can be used as new fungicides and herbicides in agriculture.

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