Aspergillus westerdijkiae polyketide synthase gene "*aoks1*" is involved in the biosynthesis of ochratoxin A

Nafees Bacha [a], Ali Atoui [a,b], Florence Mathieu [a], Thierry Liboz [a], Ahmed Lebrihi [a,*]

[a] *Département Bioprocédés et Systèmes Microbiens, Laboratoire de Génie Chimique UMR5503 (CNRS/INPT/UPS), Ecole Nationale Supérieure Agronomique de Toulouse, Institut National Polytechnique de Toulouse, 1, Avenue de l'Agrobiopôle, BP32607, 31326 Castanet Tolosan, France*
[b] *Lebanese Atomic Energy Commission-CNRS, P.O. Box 11-8281, Riad El Solh, 1107 2260 Beirut, Lebanon*

A B S T R A C T

Ochratoxin A (OTA) is a potential nephrotoxic, teratogenic, immunogenic, hepatotoxic and carcinogenic mycotoxin, produced by *Aspergillus westerdijkiae* NRRL 3174. Herein we describe the characterization of a putative OTA-polyketide synthase gene "*aoks1*", cloned by using gene walking approach. The predicted amino acid sequence of the 2 kb clone display 34–60% similarities to different polyketide synthase genes including lovastatine biosynthesis gene "*lovb*" in *A. terreus*, compactin biosynthesis gene "*mlcA*" in *Penicillium citrinum* and OTA biosynthesis gene "*otapksPN*" in *P. nordicum.* Based on the reverse transcription PCR and kinetic secondary metabolites production studies, *aoks1* expression was found to be associated with OTA biosynthesis. Further a mutant, in which the *aoks1* gene was inactivated by *Escherichia coli* hygromycin B phosphotransferase gene, lost the capacity to produce OTA, but still producing mellein. To our knowledge this report describes for the first time characterization of a gene involved in OTA biosynthesis, with the information about mellein which was proposed in the literature to be an intermediate OTA. This study also suggests that *aoks1* may be the second polyketide synthase gene required for OTA biosynthesis in *A. westerdijkiae* NRRL 3174.

1. Introduction

Ochratoxin A (OTA) is a polyketide secondary metabolite produced by many *Penicillium* and *Aspergillus* species (Abarca et al., 1994; Dalcero et al., 2002; Teren et al., 1996; Varga et al., 2003). This mycotoxin consisting of a polyketide derived from a dihydroiso-coumarin moiety linked through the 12-carboxyl group to phenylalanine, via an amide linkage (Fig. 1a). 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).

Ochratoxin A 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 (Batista et al., 2003; Battilani et al., 2003; Bayman et al., 2002; Jorgensen, 1998). The presence of OTA has been detected in a range of beverages (Zimmerli and Dick, 1996) and has also been reported in body fluids and kidneys of animals and humans (Magan and Olsen, 2004).

Aspergillus westerdijkiae NRRL 3174 which is recently dismembered from *A. ochraceus* (Frisvad et al., 2004), is considered to be the main OTA producer in tropical region. It can produce other important polyketide metabolites including; penicillic acid, asperlactone, isoasperlactone, mellein and hydroxymellein (Atoui et al., 2006; Gaucher and Shepherd, 1968). To the present day not much information are available about the biosynthetic pathway of OTA in any fungal species. Based on a mechanistical model according to the structure of OTA a biosynthetic pathway has been previously proposed, according to which the heterocyclic portion of OTA is structurally similar to mellein (Fig. 1b) (Huff and Hamilton, 1979). Thus mellein has been proposed as a precursor of OTA. In contrary, Harris and Mantle (2001) described in experiments with labeled precursors of OTA that mellein does not seem to play a role in OTA biosynthetic pathway.

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 (Birch and Donovan, 1953; Kao et al., 1994). In the biosynthesis of most polyketide metabolites, the successive condensation step of small carbon precursor acid is catalyzed by a group of multifunction enzyme system

* Corresponding author. Fax: +33 5 62 19 39 01.
E-mail address: lebrihi@ensat.fr (A. Lebrihi).

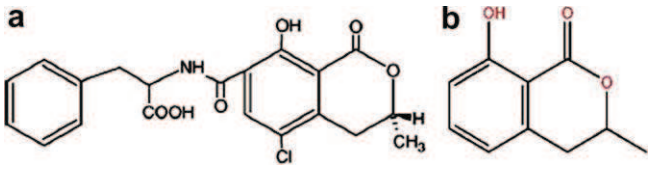

Fig. 1. Chemical structures of ochratoxin A (a) and mellein (b).

called polyketide synthases (PKSs), (Metz et al., 2001). 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) (Graziani et al., 2004). Presence or absence of optional domains in a PKS decides about the type of polyketide formed. PKSs producing highly reduced polyketides contain KR, DH and ER optional domains; PKSs producing partially reduced polyketides contain KR and DH optional domains, while PKSs producing non-reduced polyketides contain none of these domains (Fujii et al., 2001; Yu and Leonard, 1995).

Untill now different techniques like genomic DNA bank, cDNA bank and subtractive PCR has been utilized to identify various PKS genes responsible for the biosynthesis of various polyketides (Bridge et al., 1998). Pairs of degenerated primers targeting KS domain; which is the most conserved domain among different PKSs, have been previously designed. These primers were found to have the capability to amplify KS domain fragment from different types of PKS genes (Bingle et al., 1999; Lee et al., 2001; Liou and Khosla, 2003; Nicholson et al., 2001). In our laboratory these degenerated primers were used to identify nine different KS domains in *A. westerdijkiae* NRRL 3174 (= *A. ochraceus*) (Atoui et al., 2006), including the KS domain sequence of *aolc35-12* gene (Genbank Accession No. AY583208). *aolc35-12* gene overlaps the AT domain of the *pks* gene characterized by O'Callaghan et al. (2003). The authors demonstrated that disruption of *pks* gene stop the biosynthesis of OTA, without any information about mellein.

In this study, we report the characterization of a PKS gene (*aoks1*) required for the biosynthesis OTA in *A. westerdijkiae* NRRL 3174. Disruption of this gene stop the biosynthesis of OTA but did not affect the biosynthesis of mellein. This gene is different from the *pks* gene reported by O'Callaghan et al. (2003).

2. Materials and methods

2.1. Fungal strain and culture conditions

Aspergillus westerdijkiae NRRL 3174 strain provided by Dr. Olivier Puel (Laboratoire de Pharmacologie-Toxicologie, Toulouse France) was grown for sporulation at 25 °C on potato dextrose agar for 7 days. Spores were collected using a solution of 0.01% (v/v) Tween 80, counted by using Thoma Bright line counting chamber (Optick labor), and stored at −20 °C in 25% (v/v) glycerol before use. Conidia were inoculated (density $\sim 10^6$/mL) into 250 mL Erlenmeyer flasks containing 100 mL synthetic medium (SAM) at 25 °C for 2–18 days, without shaking. The composition of SAM (per liter of distilled water) was: 3 g NH_4NO_3, 26 g K_2HPO_4, 1 g KCl, 1 g $MgSO_4.7H_2O$, 10 mL mineral solution (composition per liter of distilled water: 70 mg $Na_2B_4O_7.10H_2O$, 50 mg $(NH_4)_6$ $Mo_7O_{24}.4H_2O$, 1000 mg $FeSO_4.7H_2O$, 30 mg $CuSO_4.5H_2O$, 11 mg $MnSO_4.H_2O$, 1760 mg $ZnSO_4.7H_2O$), and 50 g glucose. The pH of the medium was adjusted to 6.5 by the addition of 2 N HCl. Mycelium was harvested by filtration through a 0.45 μM filter, grounded in liquid nitrogen and then stored at −80 °C before nucleic acid extraction. Secondary metabolites were extracted from filtrates of 2 to 18 days old cultures medium. Three replications of each sample were analyzed.

2.2. Nucleic acid extraction

Rapid method of genomic DNA extraction (Lui et al., 2000) was used for transformants screening by PCR. Large quantity genomic DNA was extracted by CTAB extraction method (Gardes and Bruns, 1993). The quality and quantity of DNA were estimated by measuring OD 260 nm/OD 280 nm and OD 260 nm respectively.

Total RNA was extracted from *A. westerdijkiae* NRRL 3174 using the Tri-reagent (Euromedex France) DNA/RNA/Protein extraction kit. The quality and quantity of RNA was checked by the OD 260 nm/OD 280 nm ratio and agarose gel electrophoresis according to standard protocols (Sambrook et al., 1989).

2.3. Cloning of aoks1 gene in A. westerdijkiae NRRL 3174

Degenerated primers were used to extend *aoks1* gene by gene walking approach. Table 1 list all the primers used in this study. Fig. 2 marks position of specific primer AoKS1 (designed from the KS domain fragment of *aoks1* gene) and the two degenerated primers ATR1, ATR2 (designed from the most conserved regions of AT domains of different fungal PKSs). In two consective PCRs; first a 700 bp fragment was amplified using primers AoKS1 and ATR1 and then a 1400 bp fragment was amplified using primers AoKS1 and ATR2. The PCR products were cloned in pCR2.1 plasmid and sequenced. Alignment of the sequenced fragments was performed to search for consensus.

2.4. PCR reaction and sequencing

PCR was performed with the *Taq* recombinant polymerase (Invitrogen, USA). Amplification was carried out in a 50 μl reaction mixture containing: 5 μl of *Taq* polymerase 10× buffer, 1·5 μl of 50 mM $MgCl_2$, 1 μl of dNTP 10 mM of each (Promega), 1 μM of each primer, 1·5 U of *Taq*, about 200 ng of DNA genomic, H_2O up to 50 μl. Reaction conditions were: 94 °C for 4 min, (94 °C for 45 s, 53 °C for 45 s and 72 °C for 1 min) ×30 cycles followed by an incubation at 72 °C for 10 min. The amplified products were examined by 1% (w/v) agarose (Promega) gel. The PCR products were cloned into pCR2.1-Topo vector (Invitrogen) according to the supplier's instructions. Sequencing of the fragments was performed by Genomexpress (Grenoble, France).

2.5. Data analysis

The deduced amino acid sequence was determined using the http://www.expasy.org/tools/dna.html site while protein-protein Blast (Blastp) searches were conducted at the GenBank database: http://www.ncbi.nlm.nih.gov. The alignments were conducted using CLC Main WorkBench (CLC Bio, Denmark).

Table 1
Primers used in this study.

Primer name	Sequence (5′–3′)
ATR1	YTG5GC5CCYTG5CC5GTDAA
ATR2	CATRTGRTGIGARTGRTAIGC
AoKS1	CGGAAGGCCGGCCTAGATCCAGCC
AoKS1F	GAAGCCGTCGAGGCCGCCGGTCTG
AoKS1R	CAATGCGAATTGCCTCTATTTC
AoKSF	CGGCCAATCTGGGAGATTTGGC
hph2F	CGGGGGCAATGAGATATGAAAAAG
hph2R	GAACCCGCTCGTCTGGCTAAG
LC12F	CTATGACTTACGCGGGACAAG
LC12R	AAGGCAGATACAATGGCCTGC
TubF	CTCGAGCGTATGAACGTCTAC
TubR	AAACCCTGGAGGCAGTCGC

Fig. 2. Alignment of the deduced amino acid sequence of *A. westerdijkiae aoks1* with other PKSs: *lovB* (Accession No. AAD39830, lovastatin nonaketide synthase in *A. terreus*); *mlcA* (Accession No. BAC20564, compactin nonaketide synthase in *P. citrinum*); *otapksPN* gene (Accession No. AY557343, OTA biosynthesis gene in *P. nordicum*); *aolc35-12* (Accession No. AY583208) and *pks* (Accession No. AY272043, OTA biosynthesis gene characterized by O'Callaghan et al. (2003)) in *A. ochraceus*. Red box indicates KS domain while green box indicates AT domain. Flash arrows indicate positions of different primers used in *extanding* aoks1 gene. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

2.6. RT-PCR reaction

The expression of *aoks1* and *aolc35-12* genes in *A. westerdijkiae* NRRL 3174 were examined by using RT-PCR with specific pairs of primers AoKSF/AoKS1R designed from the AT domain of *aoks1* gene and LC12F/LC12R designed from the KS domain of *aolc35-12* gene (Table 1). For RT-PCR, total RNA was treated with DNase I (Promega) to remove DNA contamination. cDNA was synthesized from each sample with Advantage RT- for-PCR Kit (BD Biosciences) according to the supplier's manual. cDNA amplification were performed using Taq recombinant polymerase (Invitrogen,USA). Beta tubulin was used as positive control using primers TubF and TubR (Table 1).

2.7. Disruption of aoks1 gene in A. westerdijkiae NRRL 3174

The *aoks1* gene was disrupted by inserting the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*) flanked by *A. nidulans trpC* promoter and terminator sequences (Cullen et al., 1987) from plasmid pID2.1 (Tang et al., 1992). The transformation vector construction is schematically represented in Fig. 3a. A 1690 bp

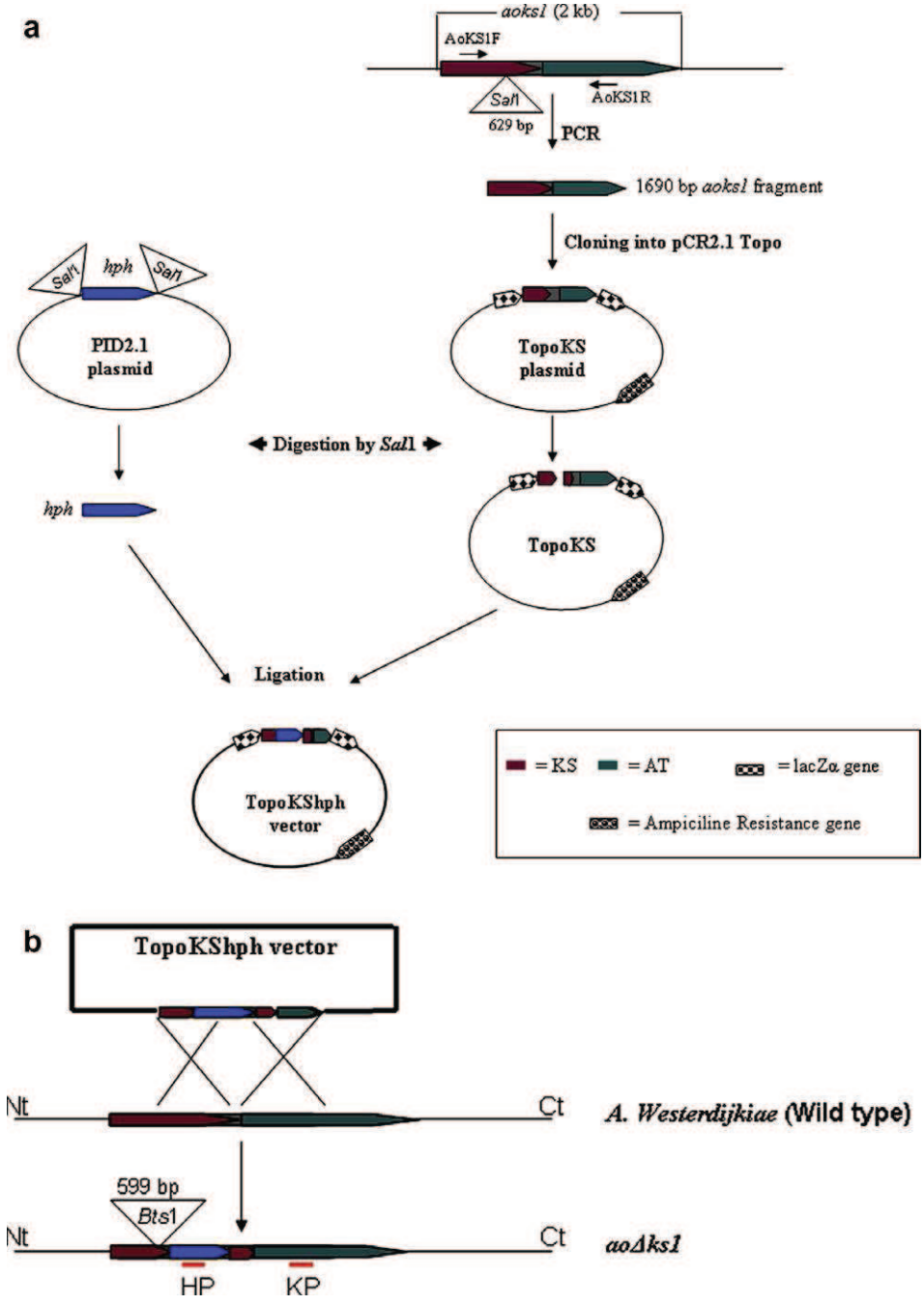

Fig. 3. Schematic representation of transformation vector formation and *aoks1* gene disruption. (a) Using primer pair AoKS1F/AoKS1R (Table 1), 1690 bp *aoks1* fragment containing Sal1 restriction site (indicated by triangle) was amplified from a 2 kb *aoks1* gene. PCR product was cloned into pCR2.1-Topo plasmid to generate plasmid TopoKS. pID2.1 plasmid vector was restricted with Sal1 (indicated by triangle) to obtain *hph* cassette (2.4 kb). TopoKS was restricted with Sal1 and ligated with *hph* cassette to genarate TopoKShph transformation vector. Different colors on the *aoks1* gene indicate different functional domains i.e. β-ketoacyl synthase (KS) and acyltransferase (AT). (b) Protoplasts of *A. westerdijkiae* was prepared and *aoks1* gene was disrupted using TopoKShph vector to obtain *aoΔks1* mutant. The small red lines below *aoΔks1* indicate position of radioactively labeled probes i.e. KP: *aoks* gene specific probe amplified by using primer pair AoKSF/AoKS1R and, HP: *hph* gene specific probe amplified by using primer pair hph2F/hph2R. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

fragment of the *aoks1* gene containing the SalI restriction site was amplified by PCR, using the primer pair AoKS1F/AoKS1R (Table 1). This fragment was ligated into pCR2.1-Topo plasmid (Invitrogen) generating the TopoKS plasmid. TopoKS and pID2.1 plasmids were restricted using SalI enzyme. The *hph* cassette (2.4 kb) obtained was ligated with restricted TopoKS plasmid to produce TopoKShph vector, in which the 5′ and 3′-ends of *hph* cassette are flancked by *aoks1* gene fragments. *A. westerdijkiae* protoplasts were prepared and transformed with TopoKShph vector as previously described (O'Callaghan et al., 2003) (Fig. 3b). Lysing enzyme (40 mg/mL) (Sigma) was used for the preparation of protoplasts.

2.8. Screening of the transformants

Hygromycin-resistant transformants were selected on YES medium (20 g/l of yeast extract, 1 M sucrose) supplemented with 150 μg/mL of hygromycin B. Transformant plates were incubated at room temperature for 24 h and then transferred to 30 °C for 4 days. Hygromycin-resistant transformants were further screened through a PCR, using *hph* gene specific primers hph2F and hph2R (Table 1). Positive transformants were then subjected to a second PCR using *aoks1* gene specific primer AoKS1F and hph2R. Finally the disruption was confirmed through southern hybridization (Southern, 1975), in which total genomic DNA from both wild type and *aoΔks1* mutant strains were digested with restriction enzyme Bts1. This restriction enzyme was choosen because it cut only once in the 5′-flanking region of the 1690 bp *aoks1* fragment (position 599 bp) but does not cut the *hph* cassette or 3′-flanking end of the *aoks1* fragment (Fig. 3b). The digested DNA was then transferred to nylon membrane (Amersham, France) and probed with radioactively labeled DNA fragments of *aoks1* gene "KP" and *hph* gene "HP" amplified through primer pair AoKSF/AoKS1R and hph2F/hph2R, respectively (probes positions are shown in Fig. 3b).

2.9. Fluoresence study

In order to check the loss of OTA production by the *aoΔks1* mutant, Czapek Yeast extract Agar (CYA) plates of both wild type *A. westerdijkiae* NRRL 3174 and *aoΔks1* mutant were incubated for 10 days at 25 °C and then illuminated with UV light (265 nm). Production of OTA is characterized by a blue fluorescent halo under UV.

2.10. Extraction of secondary metabolites

For secondary metabolites extraction, 30 mL filtrate sample of the culture medium was acidified with 200 μL of 12 N HCl, mixed with 30 mL chloroform and vigorously shaken for 10 min. The solvent phase was then decanted, dried under vacuum and re-dissolved in 0.5 mL Methanol. 20 μL of the sample was then further analysed by HPLC.

2.11. High-performance liquid-chromatography (HPLC) analysis

The HPLC apparatus consisted of a solvent delivery system, with both fluorescence (λ_{ex} = 332 nm; λ_{em} = 466 nm) and UV detectors (BIO-TEK, Milan, Italy). The analytical column used was a 150 × 4.6 mm Uptisphere 5 μm C18 ODB fitted with a guard column of 10 × 4 mm. The column temperature was 30 °C. Kroma 3000 (BIO-TEK) was the data acquisition system. Injections were done with an auto-injector (BIO-TEK, Milan, Italy) and the injection volume was 20 μL. The samples were analyzed by linear gradient elution using 0.2% glacial acetic acid in 99.8% water (v/v) (A) and 100% acetonitrile (HPLC grade) (B). The crude extract was analyzed using a linear elution gradient over 45 min at a flow rate of 1 ml/min, starting from 10 to 50% solvent B over the first 30 min, continued by a linear gradient to 90% of B in 5 min, followed by an isocratic flow of 90% solvent B for 8 min, and a return to initial conditions over the last 2 min of the run.

Secondary metabolites of *A. westerdijkiae* NRRL 3174 were detected by comparing the elution time and maximum absorption of UV with the standards (Sigma Aldrich France). All the standards were used at a concentration of 10 μg/mL. Mellein (λ_{max} = 214; 245 and 314 nm) and ochratoxin A (λ_{max} = 216; 250 and 332 nm) were released at 27 min and 34 min, respectively.

2.12. Sequence accession number

The sequence obtained was deposited in Genbank under the Accession No. AY583209.

3. Results and discussion

3.1. Cloning part of aoks1 gene in A. westerdijkiae NRRL 3174

A 700 bp KS domain fragment of *aoks1* gene was previously identified in *A. westerdijkiae* NRRL 3174 (Atoui et al., 2006). With the aim of extending this fragment of *aoks1* gene, two conserved regions **FTGQGAQ** and **AYHSSHM** in the AT domains of different fungal PKSs were used to design two degenerated primers i.e. ATR1 and ATR2 (Fig. 2). These degenerated primers with a specific primer AoKS1 i.e. designed from the KS domain of *aoks1* (Fig. 2), allowed to clone an additional 1300 bp fragment of *aoks1* gene. Atoui et al. (2006) further used the degenerated primers of Bingle et al. (1999) and Nicholson et al. (2001) and identified eight other KS domains of different PKSs in same fungi. The phylogenetic analysis of all the nine KS domain fragments identified in *A. westerdijkiae*, clustured *aoks1* gene with four other PKSs producing reduced polyketides (Atoui et al., 2006). Several mycotoxins such as OTA, viomellein, and xanthomegnin and others polyketides derived secondary metabolites such as mellein produced by *A. westerdijkiae* NRRL 3174 belong to the reduced polyketide groups. Therefore, we focused on the 5 reducing PKS genes including *aoks1* and assumed that these genes could be potential candidates for the biosynthesis of one or more of the reduced polyketides produce by *A.westerdijkiae*.

The predicted amino acid sequence of the 2 kb *aoks1* gene displayed about 60% identity to nonaketide synthases gene "*lovB*" involved in lovastatin biosynthesis in *A. terreus* (Hendrickson et al., 1999), and compactin biosynthesis gene *mlcA* in *Penicillium citrinum* (Abe et al., 2002) and 34% identity to OTA biosynthesis gene *otapksPN* in *P. nordicum* (Färber and Geisen, 2004; Karolewiez and Geisen, 2005) (Fig. 2). The AT domain of *aoks1* displayed about 39% identity to the AT domain of *pks* gene involved in OTA biosynthesis in *A. ochraceus* (O'Callaghan et al., 2003). Alignment study also revealed that the *pks* gene characterized by O'Callaghan et al. (2003) overlaps (share more than 98% identity) a PKS gene *aolc35-12*, previously identified in *A. westerdijkiae* (Fig. 2) (Atoui et al., 2006; Dao et al., 2005). This finding confirms that *A. ochraceus* strain used by O'Callaghan et al. (2003) and *A. westerdijkiae* strain used in our study contain similar gene.

3.2. Kinetic production of secondary metabolites in A. westerdijkiae NRRL 3174 and expression of aoks1 and aolc35-12 genes

Production of secondary metabolites was followed during the growth of *A. westerdijkiae* NRRL 3174 in synthetic medium (SAM). We observed that biosynthesis of secondary metabolites seemed to be associated with the delayed log phase of fungal mycelial growth (Fig. 4a). This fact has also been realized previously, where biosynthesis of various secondary metabolites were found to be associated with fungal growth and development

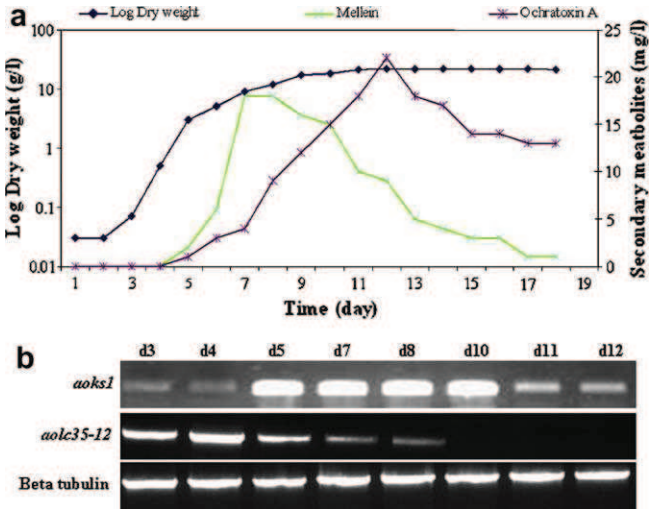

Fig. 4. Kinetic production of secondary metabolites and comparison of two PKS genes expression, in *A. westerdijkiae* NRRL 3174. (a) Kinetic production of mellein and OTA in a growing culture of *A. westerdijkiae* NRRL 3174 at 25 °C in synthetic medium during a time course of 2-18 days. (b) Profile of *aoks1* gene expression (upper panel) and *aolc35-12* gene expression (middle panel) by RT-PCR. Beta tubulin was used as loading control (bottom panel).

(Bu'Lock et al., 1968; Calvo et al., 2001, 2002; Hopwood, 1988). HPLC traces revealed that the two structurally related metabolites mellein (λ_{max} = 214; 245 and 314 nm) and ochratoxin A (λ_{max} = 216; 250 and 332 nm) are released at 27 and 34 min, respectively (Fig. 6b). Both these metabolites were detected from day 4 of fungal growth. Thereafter these metabolites were simultaneously produced to reach a maximum level at day 7 for mellein, and day 12 for OTA (Fig. 4a). Later, mellein constantly decreased with time and reached a minimum level at day 16. Small decrease in the production OTA was observed up to day 17 (Fig. 4a).

Expression study of *aoks1* gene showed that the transcriptional signal started from day 3 of *A. westerdijkiae* growth in synthetic (SAM) medium (Fig. 4b). The signal reached its maximum level at day 5 and remained high up to day 10. From expression of aoks1 gene and kinetic production of secondary metabolites we observed that, the low level expression of *aoks1* gene at day 3 and 4 could correspond to the limited production of OTA in the initial few days of fungal growth. During the high transcriptional signal period of *aoks1* gene i.e. day 5 to day 10, production of OTA continuously increased and reached its maximum limit just after the end of this period (Fig. 4a and b). These results suggest that production pattern of OTA seems to be associated with expression pattern of *aoks1* gene. On the basis of expression studies several other PKS genes producing reduced type polyketides were found to be important for OTA biosynthesis in other organisms like *P. nordicum* (Färber and Geisen, 2004; Karolewiez and Geisen, 2005) and *P. verrucosum* (Schmidt-Heydt et al., 2008).

We also studied the expression pattern of *aolc35-12* gene (similar to *pks* gene involved in OTA biosynthesis, characterized by O'Callaghan et al. (2003)) in *A. westerdijkiae*. Transcriptional signal of *aolc35-12* gene started on day 3 and reached a maximum at day 4 of fungal growth (Fig. 4b). The signals then gradually decreased and disappeared at day 10. O'Callaghan et al. (2003) also stated that expression of *pks* gene appeared strongly at the early fungal growth stage and occurred to a lesser extent at later time points. This suggests that the *pks* gene characterized by O'Callaghan et al. (2003) and *aolc35-12* not only overlap at amino acid level (Fig. 2) but also produce similar expression patterns. We further observed that, although both *aoks1* and *aolc35-12* genes are expressed within the production period of OTA but both produced different expression patterns (Fig. 4b).

3.3. Disruption of aoks1 gene in A. westerdijkiae NRRL 3174 and secondary metabolites production in ao⊿ks1 mutant

After transformation of *A. westerdijkiae* with TopoKShph vector (Fig. 3b), 27 transformants were obtained. These transformants were subsequently screend by two consective PCRs to moniter integration of *hph* cassette in the genome of *A. westerdijkiae*. A fragment of 0.6 kb corresponds to *hph* cassette is amplified using primer pair hph2F/hph2R in only two out of the 27 transformants (Fig. 5a, lane 1). Similarly a 1.2 kb *aoks1*/*hph* shared fragment was amplified when the two positive transformants were subjected to a second PCR using primers AoKS1F and hph2R (Fig. 5a, lane 4). No amplification was observed in the wild type *A. westerdijkiae* with any of the primers combination (Fig. 5a, lane 2 and 3).

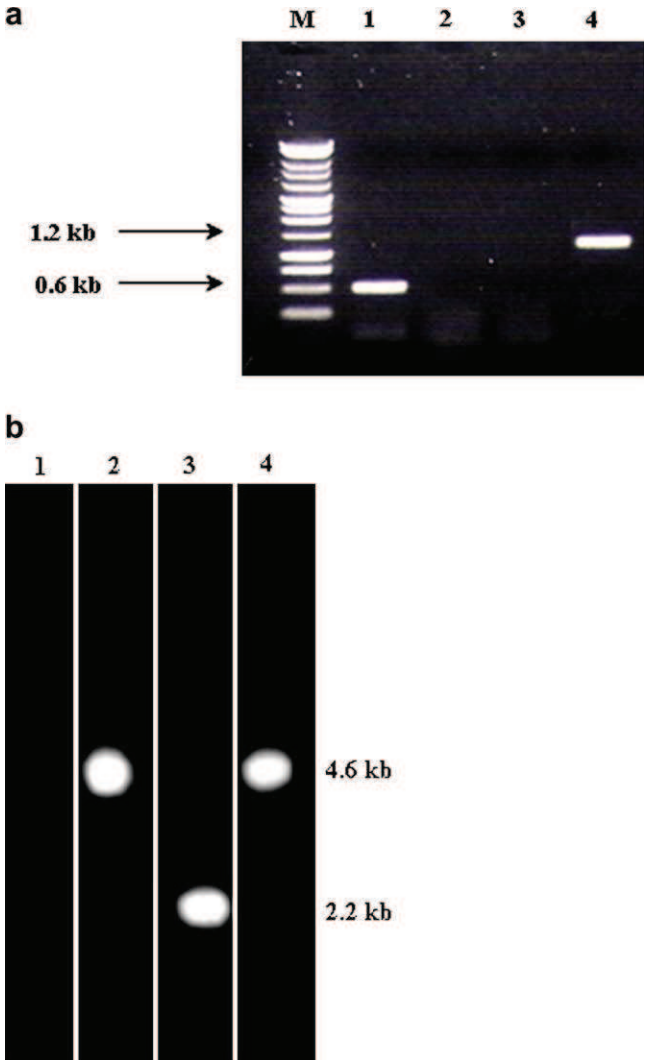

Fig. 5. PCR screening and southern blot hybridization of the transformants. (a) the PCR product of 0.6 kb (lane 1) obtained with primers hph2F/hph2R and the 1.2 kb product (lane 4) obtained with AoKS1F/hph2R confirmed the integration of the *hph* gene cassette into the genome of *A. westerdijkiae* NRRL 3174 (*ao⊿ks1* transformants). No product was amplified in *A. westerdijkiae* wild type with hph2F/hph2R (lane 2) and AoKS1F/hph2R (lane3). Lane M: size markers (Promega). (b) Southern blot screening of transformants: Genomic DNA of wild type *A. westerdijkiae* and *ao⊿ks1* transformants were extracted and digested with *Bts*1 restriction enzyme (indicated by triangle). The products were analyzed through electrophoresis and then transferred to nylon membrane. Probing of the membrane with radioactively labeled *hph* fragment (HP) resulted into; lane 1: wild type *A. westerdijkiae* and lane 2: *ao⊿ks1* mutant. Probing of the membrane with radioactively labeled *aoks1* fragment (KP) resulted into; lane 3: wild type *A. westerdijkiae* and lane 4: *ao⊿ks1* mutant.

Genomic DNA from wild type *A. westerdijkiae* and the two positive *aoΔks1* transformants were analyzed by southern blotting. No signal was detected when digested DNA of wild type *A. westerdijkiae* was probed HP (Fig. 5b, lane 1), while a signal corresponding to 2.2 kb was observed when the wild type DNA was probed KP (Fig. 5b, lane 3). On the other hand probing the digested DNA of *aoΔks1* transformants with KP and HP probes resulted into an expected signal of 4.6 kb i.e 2.2 kb aoks1 fragment + 2.4 kb hph fragment (Fig. 5b, lane 2 and 4 respectively).

Both *aoΔks1* mutant and wild type *A. westerdijkiae* NRRL 3174 were inoculated in solid CYA medium and incubated for 10 days at 25 °C. After the incubation period we observed no difference in fungal growth, sporulation, or pigment production. In contrary, *aoΔks1* mutant lost the characheristic fluorescence of OTA, when observed under UV light (Fig. 6a). This method has been usefully used by O'Callaghan et al. (2003) in order to identify OTA negative mutants.

Further, the two positive *aoΔks1* mutants were inoculated into liquid SAM medium to check kinetic production of secondary metabolites by using HPLC traces and UV spectra. Disruption of *aoks1* abolished OTA production in the *aoΔks1* mutant but did not affect the production of an important metabolite mellein (Fig. 6b). Mellein was previously thought to be an intermediate in the biosynthesis of OTA (Huff and Hamilton, 1979). Recently this hypothesis has been dissented by Harris and Mantle (2001) using labbeled precursors of OTA. They found no evidence for the intermediate role of mellein in OTA biosynthetic pathway. This is in agreement with our results, where the disruption of *aoks1* gene has been shown to interrupt the biosynthesis of OTA without affecting the mellein production. Indeed, during the kinetic

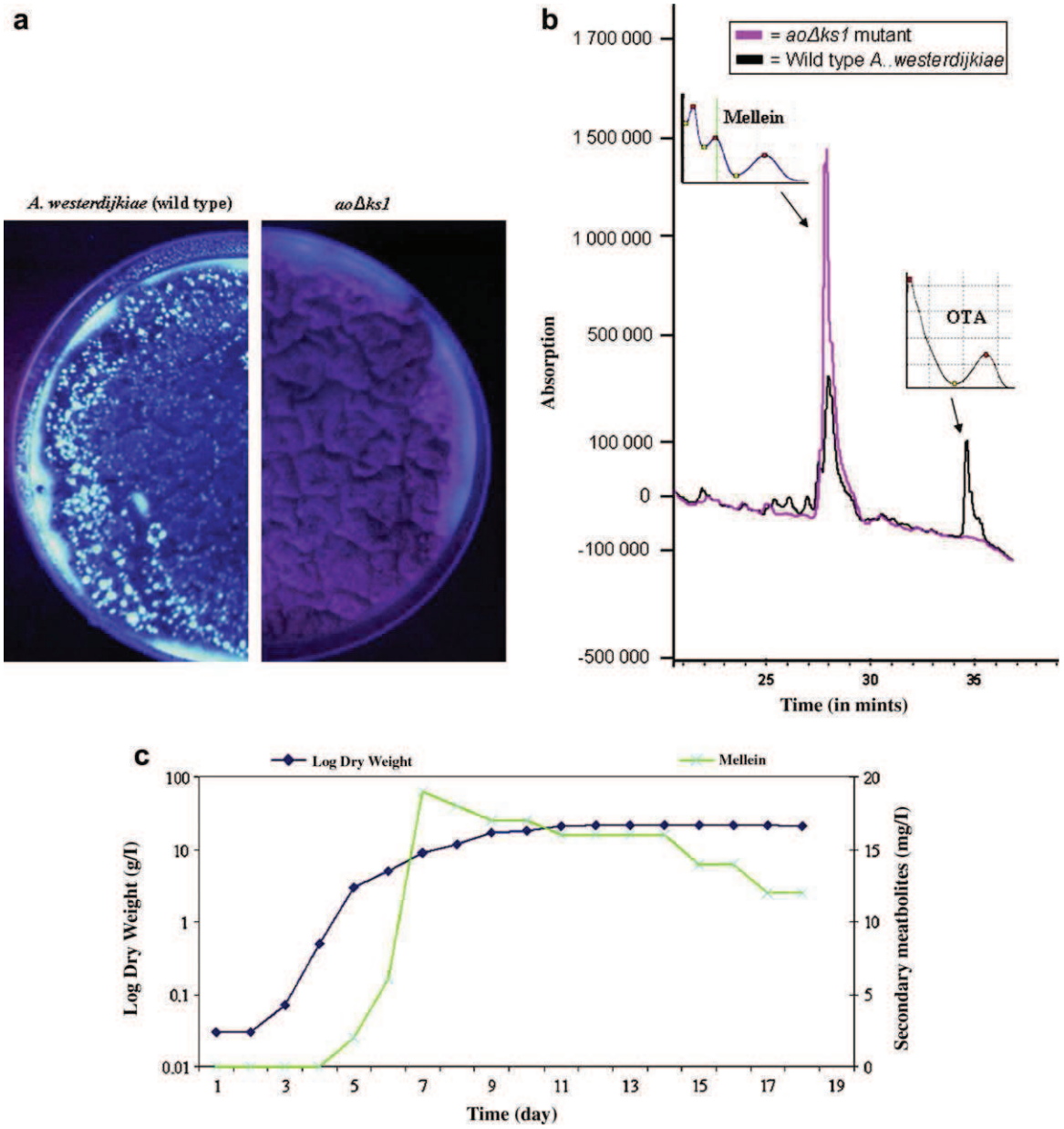

Fig. 6. Tracking secondary metabolites production in wild type *A. westerdijkiae* NRRL 3174 and *aoΔks1* mutant. (a) Characteristic fluorescence of OTA under UV light observed in wild type *A. westerdijkiae* NRRL 3174 CYA culture, and the absence of fluorescence in *aoΔks1* mutant culture. (b) HPLC traces and UV spectra of mellein (eluted at 27 min) and OTA (eluted at 34 min) in wild type *A. westerdijkiae* NRRL 3174 (black line) and *aoΔks1* mutant (pink color line). (c) Kinetic production of mellein in *aoΔks1* mutant during a time course of 2–18 days. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

production study of secondary metabolites by *aoΔks1* mutant, we observed that mellein accumulation increased after disruption of *aoks1* gene and when OTA production was halted (Figs. 4a and 6c). This could be explained by the fact that mellein and OTA use the same precursor (such as acetyl-CoA), so possibly deletion of one pathway resulted in an increase in the flow of second pathaway. In contrary, the study reported by O'Callaghan et al. (2003) about OTA biosynthesis and *pks* gene provides no information concerning the presence or absence of some metabolites like mellein.

We concluded that two different PKS may be involved in the biosynthesis of OTA and that mellein has no role in OTA biosynthesis in *A. westerdijkiae* NRRL 3174. To date, only three cases have been reported that involve two different fungal PKSs essential for a single polyketide: a set of two unusual type I multifunctional PKSs for the biosynthesis of lovastatin and compactin in *A. terreus* and *P. citrinum*, respectively (Abe et al., 2002; Hendrickson et al., 1999; Kennedy et al., 1999) and two PKS have been reported to be involved in the biosysnthesis of the mycotoxins zearaleone in *Gibberella zea* (Kim et al., 2005) and T toxin in *Cochliobolus heterosphorus* (Baker et al., 2006).

Acknowledgment

The authors are grateful to the Higher Education Commission of Pakistan for according Ph.D scholarships to Mr. Nafees Bacha.

References

Abarca, M.L., Bragulat, M.R., Castella, G., Cabanes, F.J., 1994. Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. Appl. Environ. Microbiol. 60, 2650–2652.

Abe, Y., Suzuki, T., Ono, C., Iwamoto, K., Hosobuchi, M., Yoshikawa, H., 2002. Molecular cloning and characterization of an ML-236B (compactin) biosynthetic gene cluster in *Penicillium citrinum*. Mol. Genet. Genomics 267, 636–646.

Atoui, A., Dao, P., Mathieu, F., Lebrihi, A., 2006. Amplification and diversity analysis of ketosynthase domains of putative polyketide synthase gene in *Aspergillus ochraceus* and *Aspergillus carbonarius* producers of ochratoxin A. Mol. Nutr. Food Res. 50, 448–493.

Baker, S.E., Kroken, S., Asvarak, T., Inderbitzin, P., Li, B.Y., Shi, L., Yoder, O.C., Turgeon, B.G., 2006. Two polyketide synthase-encoding genes are required for biosynthesis of the polyketide virulence factor, T-toxin, by *Cochliobolus heterostrophus*. Mol. Plant Microbe Interact. 19, 139–149.

Batista, L.R., Chalfoun, S.M., Prado, G., Schwan, R.F., Wheals, A.E., 2003. Toxigenic fungi associated with processed (green) coffee beans (*Coffea arabica* L.). Int. J. Food Microbiol. 85, 293–300.

Battilani, P., Pietri, A., Bertuzzi, A., Languasco, L., Giorni, P., Kozakiewicz, Z., 2003. Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. J. Food Prot. 66, 633–666.

Bayman, P., Baker, J.L., Doster, M.A., Michaillides, T.J., Mahony, N.E., 2002. Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. Appl. Environ. Microbiol. 68, 2326–2329.

Bingle, L.E., Simpson, T.J., Lazarus, C.M., 1999. Ketosynthase domain probes identify two subclasses of fungal polyketide synthase genes. Fungal Genet. Biol. 26, 209–223.

Birch, A.J., Donovan, F.W., 1953. Studies in relation to biosynthesis I. Some possible routes to derivatives of Orcinol and Phloroglucinol. Aust. J. Chem. 6, 360–368.

Bridge, P.D., Arora, D.K., Reddy, C.A., Elander, R.P., 1998. Applications of PCR in Mycology. CAB International, UK.

Bu'Lock, J.D., Sheperd, D., Winstanley, D.J., 1968. Regulation of 6-methylsalicylate and patulin synthesis in *Penicillium urticae*. Can. J. Microbiol. 15, 279–285.

Calvo, A.M., Gardner, H.W., Keller, N.P., 2001. Genetic connection between fatty acid metabolism and sporulation in *Aspergillus nidulans*. J. Biol. Chem. 276, 20766–20774.

Calvo, A.M., Wilson, R.A., Bok, J.W., Keller, N.P., 2002. Relationship between secondary metabolism and fungal development. Microbiol. Mol. Biol. Rev. 66, 447–459.

Cullen, D., Leong, S.A., Wilson, L.J., Henner, D.J., 1987. Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, hph. Gene 57, 21–26.

Dalcero, A., Magnoli, C., Hallak, C., Chiacchiera, S.M., Palacio, G., Rosa, C.A., 2002. Detection of ochratoxin A in animal feeds and capacity to produce this mycotoxin by *Aspergillus* section Nigri in Argentina. Food Addit. Contam. 19, 1065–1072.

Dao, H.P., Mathieu, F., Lebrihi, A., 2005. Two primer pairs to detect OTA producers by PCR method. Int. J. Food Microbiol. 104, 61–67.

Färber, P., Geisen, R., 2004. Analysis of differentially-expressed ochratoxin a biosynthesis genes of *Penicillium nordicum*. Eur. J. Plant Pathol. 110, 661–669.

Frisvad, J.C., Frank, J.M., Houbraken, J.A.M.P., Kuijpers, A.F.A., Samson, R.A., 2004. New ochratoxin A producing species of *Aspergillus* section Circumdati. Study Mycol. 50, 23–43.

Fujii, I., Watanabe, A., Sankawa, U., Ebizuka, Y., 2001. Identification of Claisen cyclase domain in fungal polyketide synthase WA, a naphthopyrone synthase of *Aspergillus nidulans*. Chem. Biol. 8, 189–197.

Gardes, M., Bruns, T.D., 1993. TS primers with enhanced specificity for Basidiomycetes-applicatin to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113–118.

Gaucher, G.M., Shepherd, M.G., 1968. Isolation of orsellinic acid synthase. Biochem. Biophys. Res. Com. 32, 664–671.

Graziani, S., Vasnier, C., Daboussi, M.J., 2004. Novel polyketide synthase from *Nectria haematococca*. Appl. Environ. Microbiol. 70, 2984–2988.

Harris, J.P., Mantle, P.G., 2001. Biosynthesis of ochratoxins by *Aspergillus ochraceus*. Phytochemistry 58, 709–716.

Hendrickson, L., Davis, C.R., Roach, C., Nguyen, D.K., Aldrich, T., McAda, P.C., Reeves, C.D., 1999. Lovastatin biosynthesis in *Aspergillus terreus*: characterization of blocked mutants, enzyme activities and a multifunctional polyketide synthase gene. Chem. Biol. 6, 429–439.

Hopwood, D.A., 1988. Towards an understanding of gene switching in *Streptomyces*, the basis of sporulation and antibiotic production. T. Proc. R. Soc. Lond. 235, 121–138.

Huff, E.E., Hamilton, P., 1979. Mycotoxin-their biosynthesis in fungi: ochratoxin metabolites of combined pathways. J. Food Port. 42, 815–820.

Jorgensen, K., 1998. A survey of pork, poultry, coffee, beer and pulses for Ochratoxin A. Food Addit. Contam. 15, 550–554.

Jorgensen, K., Jacobsen, J.S., 2002. Occurrence of ochratoxin A in Danish wheat and rye, 1992–1999. Food Addit. Contam. 19, 1184–1189.

Kao, C.M., Katz, L., Khosla, C., 1994. Engineered biosynthesis of a complete macrolactone in a heterologous host. Science 265, 509–512.

Karolewiez, A., Geisen, R., 2005. Cloning a part of the ochratoxin A biosynthetic gene cluster of *Penicillium nordicum* and characterisation of the ochratoxin polyketide synthase gene. Syst. Appl. Microbiol. 28, 588–595.

Kennedy, J., Auclair, K., Kendrew, S.G., Park, C., Vederas, J.C., Hutchinson, C.R., 1999. Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. Science 284, 1368–1372.

Kim, Y.T., Lee, Y.R., Jin, J., Han, K.H., Kim, H., Kim, J.C., Lee, T., Yun, S.H., Lee, Y.W., 2005. Two different polyketide synthase genes are required for synthesis of zearalenone in *Gibberella zea*. Mol. Microbiol. 58, 1102–1113.

Kuiper-Goodman, T., Scott, P.M., 1989. Review: risk assessment of the mycotoxin ochratoxin A. Biomed. Environ. Sci. 2, 179–248.

Lee, T., Yun, S.H., Hodge, K.T., Humber, R.A., Krasnoff, S.B., Turgeon, G.B., Yoder, O.C., Gibson, D.M., 2001. Polyketide synthase genes in insect- and nematode-associated fungi. Appl. Microbiol. Biotechnol. 56, 181–187.

Liou, G.F., Khosla, C., 2003. Building-block selectivity of polyketide synthases. Curr. Opin. Chem. Biol. 7, 279–284.

Lui, D., Caloe, C., Biard, R., Pedersen, J., 2000. Rapid mini preparation of fungal DNA for PCR. J. Clin. Microbiol. 38, 471.

Magan, N., Olsen, M., 2004. Mycotoxins in Food: Detection and Control. DC/Woodhead Publishing Limited, Cambridge.

Metz, J.G., Roessler, P., Facciotti, D., Levering, C., Dittrich, F., Lassner, M., Valentine, R., Lardizabal, K., Domergue, F., Yamada, A., Yazawa, K., Knauf, V., Brows, J., 2001. Production of polyunsaturated fatty acids by polyketide synthase in both prokaryotes and eukaryotes. Science 293, 290–293.

Nicholson, T.P., Rudd, B.A.M., Dawson, M., Lazarus, C.M., Simpson, T.J., Cox, R.J., 2001. Design and utility of oligonucleotide gene probes for fungal polyketide synthases. Chem. Biol. 8, 157–178.

O'Callaghan, J.J., Caddick, M.X., Dobson, A.D.W., 2003. A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. Microbiology 149, 3485–3491.

Petzinger, E., Ziegler, K., 2000. Ochratoxin A from a toxicological perspective. J. Vet. Pharmacol. Ther. 23, 91–98.

Sambrook, J., Fritsch, E.F., Maniatis, T. (Eds.), 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor laboratory press, Cold Spring Harbor.

Schmidt-Heydt, M.M., Richter, W., Michulec, M., Buttinger, G., Geisen, R., 2008. Comprehensive molecular system to study the presence, growth and ochratoxin A biosynthesis of *Penicillium verrucosum* in wheat. Food Addit. Contam. 25, 989–996.

Southern, E.M., 1975. Detection of specific sequences among DNA fragments separated by get electrophoresis. J. Mol. Biol. 98, 503–517.

Tang, C.M., Cohen, J., Holden, D.W., 1992. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. Mol. Microbiol. 6, 1663–1671.

Teren, J., Varga, J., Hamari, Z., Rinyu, E., Kevei, F., 1996. Immunochemical detection of ochratoxin A in black *Aspergillus* strains. Mycopathologia 134, 171–176.

Varga, J., Rigo, K., Kocsube, S., Farkas, B., Pal, K., 2003. Diversity of polyketide synthase gene sequences in *Aspergillus* species. Res. Microbiol. 154, 593–600.

Yu, J.H., Leonard, T.J., 1995. Sterigmatocystin biosynthesis in *Aspergillus nidulans* requires a novel type I polyketide synthase. J. Bacteriol. 177, 4792–4800.

Zimmerli, B., Dick, R., 1996. Ochratoxin A in table wine and grape-juice. Occurrence and risk assessment. Food Addit. Contam. 13, 655–668.