

# New Insight into the Ochratoxin A Biosynthetic Pathway through Deletion of a Nonribosomal Peptide Synthetase Gene in *Aspergillus carbonarius*

Antonia Gallo,<sup>a</sup> Kenneth S. Bruno,<sup>b</sup> Michele Solfrizzo,<sup>a</sup> Giancarlo Perrone,<sup>a</sup> Giuseppina Mulè,<sup>a</sup> Angelo Visconti,<sup>a</sup> and Scott E. Baker<sup>b</sup>

Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Bari, Italy,<sup>a</sup> and Chemical and Biological Process Development Group, Pacific Northwest National Laboratory, Richland, Washington, USA<sup>b</sup>

Ochratoxin A (OTA), a mycotoxin produced by *Aspergillus* and *Penicillium* species, is composed of a dihydroisocoumarin ring linked to phenylalanine, and its biosynthetic pathway has not yet been completely elucidated. Most of the knowledge regarding the genetic and enzymatic aspects of OTA biosynthesis has been elucidated in *Penicillium* species. In *Aspergillus* species, only *pk*s genes involved in the initial steps of the pathway have been partially characterized. In our study, the inactivation of a gene encoding a nonribosomal peptide synthetase (NRPS) in OTA-producing *A. carbonarius* ITEM 5010 has eliminated the ability of this fungus to produce OTA. This is the first report on the involvement of an *nrrps* gene product in OTA biosynthetic pathway in an *Aspergillus* species. The absence of OTA and ochratoxin  $\alpha$ , the isocoumarin derivative of OTA, and the concomitant increase of ochratoxin  $\beta$ , the dechloro analog of ochratoxin  $\alpha$ , were observed in the liquid culture of transformed strain. The data provide the first evidence that the enzymatic step adding phenylalanine to polyketide dihydroisocoumarin precedes the chlorination step to form OTA in *A. carbonarius* and that ochratoxin  $\alpha$  is a product of hydrolysis of OTA, giving an interesting new insight into the biosynthetic pathway of the toxin.

Ochratoxin A (OTA), initially described as a toxic metabolite of *Aspergillus ochraceus* (53), is a secondary metabolite produced by *Aspergillus* and *Penicillium* species. The economic impact of this mycotoxin is significant due to the fact that it is found as a contaminant in a wide variety of food commodities. In particular, *Penicillium verrucosum* is mainly responsible for OTA contamination of cereal-based products, *Penicillium nordicum* is responsible for contamination of some dry-cured foods, *Aspergillus ochraceus*, *Aspergillus carbonarius*, *Aspergillus westerdijkiae*, and *Aspergillus steynii* are responsible for contamination of coffee, cocoa, spices, and dried fruits, while contamination of grapes, grape juice, dried vine fruits, must, and wine is due mainly to *A. carbonarius* and, to a lesser extent, to *Aspergillus niger* “aggregate” species (21, 35, 36, 44, 48, 52). Ochratoxin A contamination of grapes constitutes a serious health and economic problem not only in southern European countries, but also in other areas of the world with Mediterranean-type climates (7, 9, 38, 44). Ochratoxin A is a potent nephrotoxin that also displays hepatotoxic, teratogenic, immunosuppressive, and carcinogenic effects (1, 17, 18, 19, 46, 47); it has been classified as a class 2B human carcinogen by the International Agency for Research on Cancer (IARC) (30). At present, wine is considered, after cereals, the second major source of OTA, and strict European Union regulation has been established, setting maximum levels for OTA of 2  $\mu\text{g kg}^{-1}$  in wine, musts, and grape juice and 10  $\mu\text{g kg}^{-1}$  for dried vine fruits (Commission regulation no. 1881/2006). Structurally, OTA comprises a dihydrocoumarin moiety linked to a molecule of L- $\beta$ -phenylalanine (Phe), derived from the shikimic acid pathway, by an amide bond. Several related compounds were also reported to occur in OTA-producing organism cultures, such as the dechlorinated analog ochratoxin B (OTB), the isocoumarin nucleus of OTA ochratoxin  $\alpha$  (OT $\alpha$ ) and its dechlorinated analogue ochratoxin  $\beta$  (OT $\beta$ )—which are not linked to phenylalanine, methyl and ethyl esters, including ochratoxin C (OTC), which is an ethyl ester de-

rivative of OTA—and several amino acid analogues (39, 40, 57). Unlike other important mycotoxins, the biosynthesis pathway of OTA has not yet been completely elucidated in detail. However, it is clear that the pathway involves some crucial steps, such as the biosynthesis of the isocoumarin group through the catalyzing action of a polyketide synthase (PKS), its ligation with the amino acid phenylalanine through the carboxyl group in a reaction catalyzed by a peptide synthetase, and the chlorination step, but the order of the reactions is not yet well defined. In this regard, several schemes have been proposed that have resulted from different studies. Huff and Hamilton (29) suggested a role in the polyketide synthesis for mellein, which would be carboxylated to OT $\beta$  and then transformed through a chloroperoxidase to OT $\alpha$ . According to their hypothesis, in the subsequent step, OT $\alpha$  would be phosphorylated and linked to the ethyl ester of phenylalanine to generate OTC, and then a deesterification would lead to the final product OTA (Fig. 1). Precursor feeding experiments with *A. ochraceus*, carried out by Harris and Mantle (27), suggested that one, possibly dominant, biosynthetic pathway involves the passage from OT $\beta$  to OT $\alpha$  and then to OTA, with a chlorinating step prior to the ligation of the polyketide (OT $\alpha$ ) to phenylalanine, without OTC and phenylalanine ethyl ester intermediates. Since they could not rule out the role of OTB, they also maintain a possible alternative pathway in which the formation of OTA goes through the synthesis from OT $\beta$  to OTB, but in this case, a bio-

Received 13 August 2012 Accepted 10 September 2012

Published ahead of print 14 September 2012

Address correspondence to Antonia Gallo, antonia.gallo@ispa.cnr.it.

Supplemental material for this article may be found at <http://aem.asm.org/>.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.02508-12

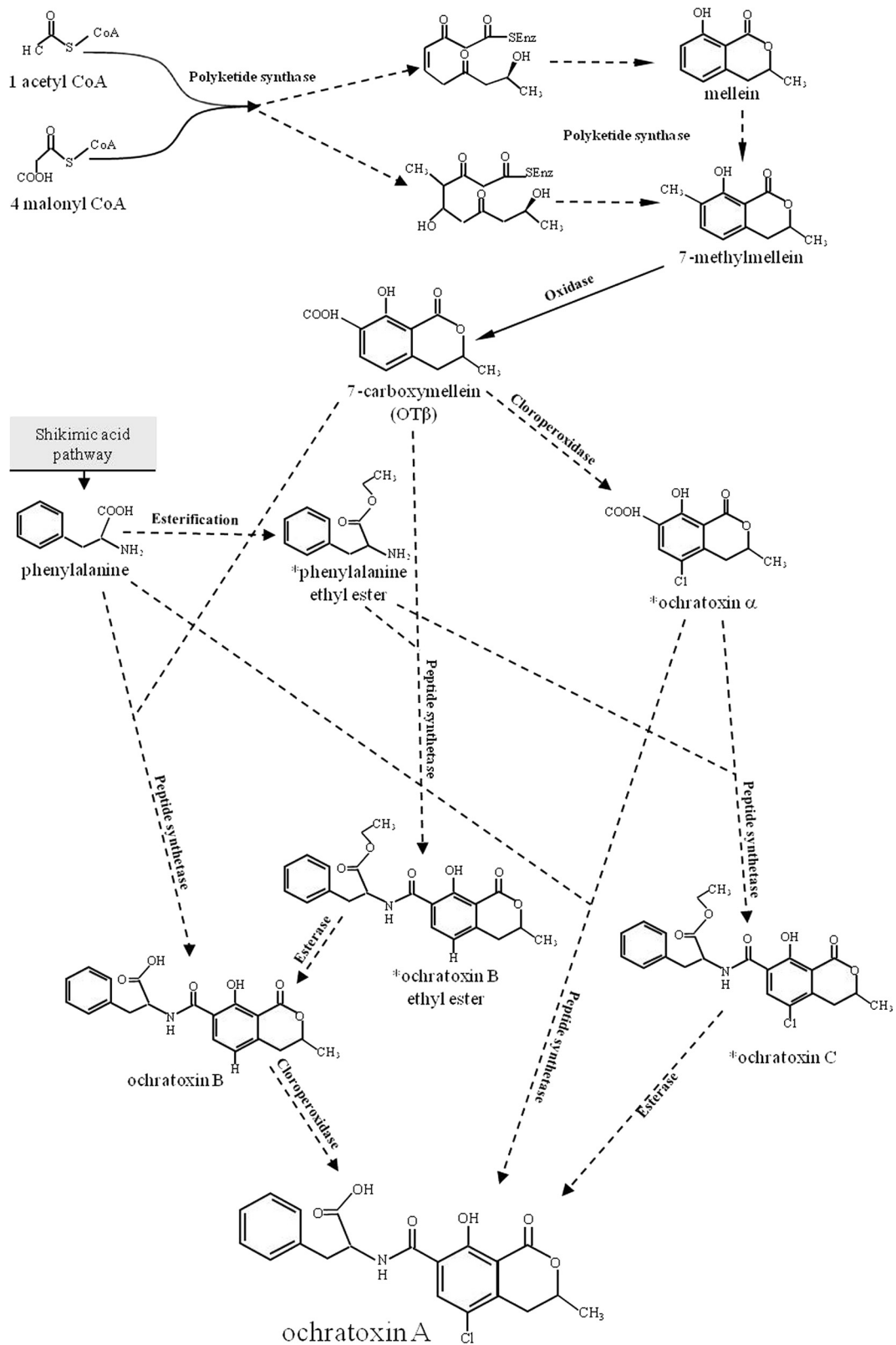


FIG 1 Scheme showing all the different hypotheses of the OTA biosynthesis pathway according to the literature data available so far (27, 29). \*, hypothetical intermediary compounds.

synthetic role for OT $\alpha$ , which occurs naturally, could not be explained. All of the hypothetical pathways for OTA biosynthesis are summarized in Fig. 1, in which the esterification or not of phenylalanine and the consequent presence of OTB ethyl ester with an intermediary role are also contemplated.

Most of the molecular aspects of OTA biosynthesis have been elucidated in *Penicillium* species. In *P. nordicum*, a putative OTA biosynthetic cluster has been identified containing biosynthetic genes encoding (i) a PKS (*otapks*PN), (ii) a nonribosomal peptide synthetase (NRPS) (*otanps*PN) putatively responsible for the formation of the peptide bond between the polyketide and the phenylalanine, (iii) a gene (*otachl*PN) with some homology to chlorinating enzymes, thought to be involved in the chlorination step, and (iv) a gene (*otatra*PN) with high homology to a transporter protein hypothesized to be involved in OTA export (25, 31). The orthologs of these genes have also been identified for the closely related species *P. verrucosum*, but have not yet been identified in the major OTA-producing aspergilli. So far, in *Aspergillus* spp., specifically in *A. ochraceus* (41) and in *A. westerdijkiae* (6), only the *pks* genes involved in the initial steps of the pathway have been characterized. In *A. carbonarius*, an economically and scientifically interesting species due to its predominant role in the contamination of grapes, the presence of different *pks* genes was investigated (5). In our previous work, we identified an additional *pks* gene in the same species whose expression pattern showed a correlation to OTA production under permissive conditions (22). In order to identify genes and proteins related to OTA biosynthesis in *A. carbonarius*, other studies were performed based on differential expression profiles, but no OTA homologous genes were found (10, 13, 14).

Recently, the genome sequence of *A. carbonarius* strain ITEM 5010, which is capable of producing up to 7.5 mg of OTA per liter of inductive culture medium (43), was generated by the United States Department of Energy's Joint Genome Institute (<http://jgi.doe.gov/carbonarius/>). Similar to many other ascomycetes so far sequenced, the *A. carbonarius* genome contains a large number of *pks* and *nmps* genes. These genes encode complex and multifunctional proteins involved in the biosynthesis of most of the fungal secondary metabolites (11, 12, 32). In particular, a new *pks* gene (protein identification [PI] no. 173482) was identified which showed a similarity of about 70% at the nucleotide level and 74% at the amino acid level to the predicted OTA cluster *pks* gene (XP001397313) of the OTA-producing *A. niger* strain CBS 513.88 (42). The percentages of identity to *A. ochraceus* (AY272043) and *A. westerdijkiae* (AY583209) OTA PKS partial sequences were 63.7% and 37%, respectively, whereas an amino acid identity of 38.7% was observed to the PKS sequence previously identified in *A. carbonarius* (22). Furthermore, it showed an identity of 30.4% to the partial sequence of *P. nordicum* OTA PKS (AY557343).

Interestingly, *A. niger* strain ATCC 1015, which does not produce OTA, has a 21-kb deletion in this predicted biosynthetic cluster (4). Similar to most characterized mycotoxins, OTA biosynthetic genes in *A. carbonarius* are expected to be arranged in a cluster. The genomic region, located by the OTA putative *pks* gene in this fungus, was investigated, leading to the identification of a gene encoding an NRPS hypothesized to catalyze the ligation of phenylalanine to the polyketide group. The gene arrangement of putative OTA clusters in different fungal species is shown in Fig. S1 in the supplemental material.

In this work, we present the inactivation of the gene in *A. car-*

*bonarius* designated *AcOTAnrps*, which completely inhibited the production of OTA, OTB, and OT $\alpha$ . The principal secondary metabolites involved in OTA biosynthetic pathway were identified in wild-type and transformant strains by high-resolution mass spectrometry (HRMS). This result indicates for the first time the involvement of an NRPS in the OTA biosynthetic pathway of an *Aspergillus* species. The absence of OTA, OTB, and OT $\alpha$  and the concomitant increase of OT $\beta$  concentration in the culture of  $\Delta$ *AcOTAnrps* strain confirm the hypothesis that the bond between the phenylalanine and the polyketide dihydroisocoumarin, catalyzed by the synthetase, precedes the chlorination step, clarifying the order of reactions in the OTA biosynthetic pathway. Moreover, we present evidence that the non-OTA-producing  $\Delta$ *AcOTAnrps* strain keeps the capability to degrade OTA in OT $\alpha$ .

## MATERIALS AND METHODS

**Fungal strains and growth conditions.** The *A. carbonarius* strain used in this study is the wild-type strain ITEM 5010 from the Agri-Food Toxicogenic Fungi Culture Collection of the Institute of Sciences of Food Production, CNR, Bari, Italy ([www.ispa.cnr.it/Collection](http://www.ispa.cnr.it/Collection)). The other two strains used in this study were generated by the wild-type strain and are indicated as KB1039 ( $\Delta$ *kusA*) and KB1041 ( $\Delta$ *kusA*  $\Delta$ *AcOTAnrps*). The mutant strains were grown on minimal medium supplemented with hygromycin (100  $\mu$ g ml<sup>-1</sup>) and/or glufosinate (Basta; bioWorld, Dublin, OH) (1 mg ml<sup>-1</sup>) for selection of transformants.

To check the production of OTA and other secondary metabolites, conidia (10<sup>5</sup> spores in 100  $\mu$ l of water) were inoculated into Erlenmeyer flasks containing 20 ml of minimal medium (6 g/liter NaNO<sub>3</sub>, 0.52 g/liter KCl, 0.52 g/liter MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1.52 g/liter KH<sub>2</sub>PO<sub>4</sub> [pH 6.5], 10 g/liter glucose, 2 ml/liter Hutner's trace elements). Incubation was carried out at 25°C in the dark under stationary conditions for 14 days. After 7 and 14 days, the fungal biomass was collected, filtered on Whatman paper no. 4 (Whatman International, Ltd., Maidstone, United Kingdom), and weighed. Sporulation and pigmentation of fungal colonies were evaluated by visual observation. Liquid cultures were collected and analyzed for OTA and OT $\alpha$  content by high-pressure liquid chromatography with fluorescence detection (HPLC-FLD). Liquid cultures of transformants KB1039 and KB1041 were analyzed by HPLC-HRMS to confirm the results of HPLC-FLD and to identify other metabolites belonging to the OTA biosynthetic pathway.

For kinetic studies, the KB1041 strain was grown for 25 days at 25°C in 100 ml of minimal medium supplemented with OTA (1.2  $\mu$ g ml<sup>-1</sup> [3  $\mu$ M]). As a control, the same amount of OTA was added to minimal medium not inoculated with the fungus. Every day, 1 ml of liquid culture was collected from each replicate and stored at -20°C until the HPLC-FLD determination of OTA and OT $\alpha$  content. Triplicate cultures were prepared and analyzed for each experiment.

**Deletion of the *AcOTAnrps* gene in *A. carbonarius*.** A deletion cassette for *AcOTAnrps* was designed and transformed into strain KB1039 of *A. carbonarius* ITEM 5010 lacking the *kusA* gene, which is required for nonhomologous end joining (NHEJ) double-strand-break repair of DNA, to facilitate homologous integration (M. Praseuth, A. Gallo, S. E. Baker, J. R. Collett, C. C. Wang, and K. S. Bruno, submitted for publication). The deletion cassette was generated by a commonly used PCR approach (58) in which sequences flanking the coding region of the *AcOTAnrps* gene were fused to the *bar* gene encoding glufosinate resistance. The primers used to create the deletion cassette are listed in Table 1. In particular, two ~1,200-bp fragments, one upstream and one downstream of the targeted gene, were amplified from the genomic *A. carbonarius* DNA with the primer pairs OTAnrpsR6/OTAnrpsF4 and OTAnrpsR3/OTAnrpsF1, respectively. A fusion PCR attached the two fragments to the *bar* resistance marker, which was amplified from the plasmid pCB1530 (Fungal Genetics Stock Center, Kansas City, MO) by using the primer pair uBarR/uBarF, which have extension sequences over-

TABLE 1 Primers used in this study

Primer name	Primer sequence (5'→3')
OTAnrpsF1	5'-AATCTCCGACAGCTGGAAAA-3'
OTAnrpsF2	5'-CTTTGGGCAGAGTTTGAAG-3'
OTAnrpsR3	5'-TGACCTCCACTAGCTCCAGCGCAAT CCGCCTTACTTTCA-3'
OTAnrpsF4	5'-AATAGAGTAGATGCCGACCGGTGG AAAGTGGGAAATGGTG-3'
OTAnrps5R	5'-GGGAATGTCTTTCGCCTTGA-3'
OTAnrps6R	5'-CTGACTGCGTAGCCTCTCCT-3'
uBarF	5'-GCTGGAGCTAGTGGAGGTCAGTC GACAGAAGATGATATTG-3'
uBarR	5'-CGTCCGCATCTACTCTATTGTC GACCTAAATCTCGGTGA-3'
AcNRPS-1for	5'-TCATCTCCGACGAGGAAC-3'
AcNRPS-2rev	5'-CAAAGGAATCCTCGTCACT-3'

lapping extension sequences of primers OTAnrpsF4 and OTAnrpsR3. The joined product was amplified with the nested primers OTAnrpsR5 and OTAnrpsF2 to obtain the construct, which was transformed into protoplasts prepared from strain KB1039 (Fig. 2A). Colonies were se-

lected for stable integration of the cassette on minimal medium supplemented with 1 mg/ml glufosinate (bioWorld, Dublin, OH). The transformants were first screened for correct integration by diagnostic PCR using the primers uBarR and OTAnrpsF1. This pair of primers can only amplify a fragment from transformants that have integrated the *bar* gene at the targeted locus. All three of the obtained transformants tested positive for proper integration. To further screen for the deletion, we amplified the entire locus using primer OTAnrpsR6 and OTAnrpsF1. These primers are outside the construct that was used to transform the parent strain. The  $\Delta kusA$  parental strain had an amplicon larger than 8 kb, and the transformants had an amplicon of about 3.4 kb (see Fig. S2 in the supplemental material). These matched the predicted sizes and were consistent with the predicted changes that should have occurred at that locus. A final confirmation of the gene deletion was made by Southern blotting, by using standard methods for DNA electrophoresis and blotting (37). Southern analysis was performed by digesting the genomic DNA of the strains with BamHI and PstI and by hybridization with a 1,586-bp probe, amplified with primers AcNRPS-2rev and OTAnrpsF1. The probe was labeled and developed using a North2South chemiluminescent detection kit (Thermo Fisher Scientific). The blot was imaged using a Kodak MR2000 equipped with a charge-coupled device (CCD) camera. Markers were visualized for size determination on the blot by loading either the Invitrogen 1-kb-plus

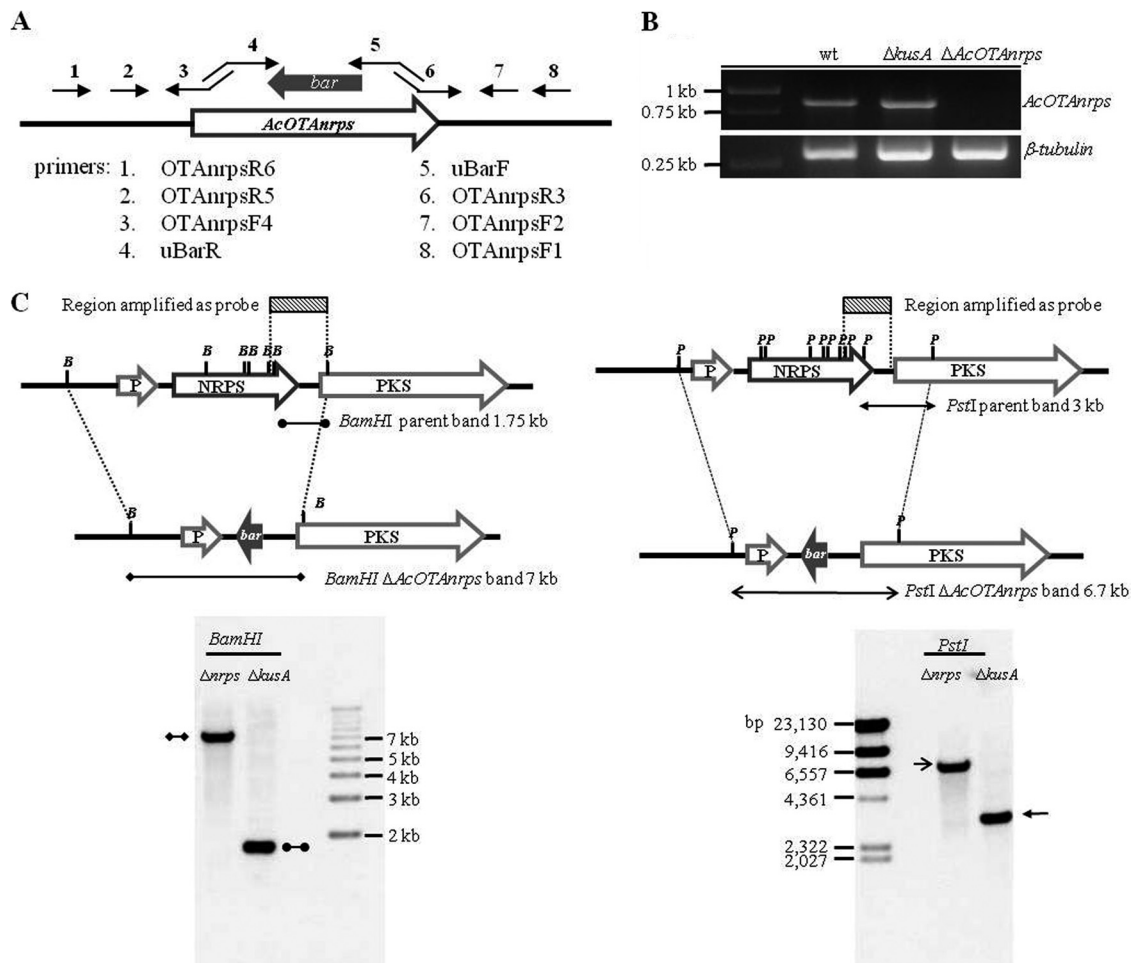


FIG 2 (A) Schematic representation of the *AcOTAnrps* gene deletion construct. (B) RT-PCR profile of *AcOTAnrps* gene expression in the wild-type,  $\Delta kusA$ , and  $\Delta AcOTAnrps$  strains grown on a medium permissive for OTA.  $\beta$ -Tubulin was used as a control. (C) Map of the *AcOTAnrps* locus in parental and  $\Delta AcOTAnrps$  strains of *A. carbonarius* and Southern blot hybridizations from genomic DNA digested with BamHI and PstI. The restriction sites BamHI and PstI are indicated with B and P, respectively.



DNA size marker or HindIII-digested lambda DNA into the outside lanes. These bands were detected using labeled lambda DNA.

**Nucleic acid extraction, cDNA synthesis, and RT-PCR.** 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 pulverized in liquid nitrogen using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA samples were treated with RNase-free DNase I (Qiagen) to eliminate possible trace amounts of contaminating DNA. RNA aliquots were preserved at  $-80^{\circ}\text{C}$ . First-strand cDNA was synthesized using about 1.5  $\mu\text{g}$  of total RNA, oligo(dT)<sub>18</sub> primer, and SuperScript III reverse transcriptase (Invitrogen, San Diego, CA) according to the manufacturer's protocol.

Reverse transcription (RT)-PCR was used to analyze the presence of *nrps* transcript in the mycelium of wild-type strain ITEM 5010 and the mutant strains KB1039 and KB1041. The primer pair AcNRPS-1for/2rev (Table 1) was used under the following conditions:  $94^{\circ}\text{C}$  for 5 min, followed by 35 cycles of  $94^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 50 s, and  $72^{\circ}\text{C}$  for 50 s, and then a final extension step of  $72^{\circ}\text{C}$  for 5 min. The primers Bt2a/Bt2b (26) were used to monitor  $\beta$ -tubulin gene expression as endogenous control. These primers span three introns, which also allowed checking for DNA contamination.

**Chemical analyses of liquid cultures by HPLC-FLD and HPLC-HRMS.** For OTA and OT $\alpha$  determination in liquid cultures of the three strains of *A. carbonarius* ITEM 5010 (wild type, KB1039, and KB1041), aliquots of liquid cultures were filtered through a 0.22- $\mu\text{m}$ -pore filter (Sartorius AG, Goettingen, Germany), and 100  $\mu\text{l}$  was injected into the HPLC-FLD apparatus. Direct injection of liquid culture was possible since no interfering peaks eluted at retention times of OT $\alpha$  and OTA. The limit of detection of this method for OTA and OT $\alpha$  was 0.1 ng/ml. This methodology was also used to measure OTA and OT $\alpha$  in liquid cultures for the kinetics study of OTA degradation by the  $\Delta\text{AcOTAnrps}$  strain. Samples containing high OTA concentrations exceeding the calibration range of the OTA standard were appropriately diluted with the HPLC mobile phase and reanalyzed.

To confirm the lack of OTA production by *A. carbonarius* KB1041 at low concentration, aliquots of liquid cultures were purified and concentrated through OchraTest immunoaffinity (IMA) columns (Vicom L.P., Watertown, MA) prior to HPLC-FLD determination. In particular, 5 ml of liquid culture was diluted with a solution containing 1% polyethylene glycol-5%  $\text{NaHCO}_3$  and loaded onto the IMA column, which was eluted at 1 ml/min by discarding the eluate. The column was then washed with 5 ml washing solution containing 2.5%  $\text{NaCl}$ , 0.5%  $\text{NaHCO}_3$ , and 5 ml pure water. The column was eluted with 2 ml methanol that was collected in a vial, evaporated to dryness at  $50^{\circ}\text{C}$  under a nitrogen stream, and reconstituted with 250  $\mu\text{l}$  of the HPLC mobile phase. Fifty microliters of purified extract, equivalent to 1 ml of liquid culture, was injected into the HPLC-FLD apparatus. The limit of detection of this method for OTA was 0.005 ng/ml. OT $\alpha$  could not be measured by this method because the IMA column has a poor retention for this compound, which is lost during sample loading on the column.

The HPLC-FLD apparatus was an 1100 series liquid chromatography (LC) system comprising a binary pump, an autosampler, a fluorescence detector (excitation wavelength, 333 nm; emission wavelength, 460 nm) from Agilent Technologies (Waldbronn, Germany). The column was a Waters Symmetry C<sub>18</sub> column (150 mm by 4.6 mm, 5- $\mu\text{m}$  particles) (Waters, Milford, MA), preceded by a 0.5- $\mu\text{m}$  Rheodyne guard filter. The mobile phase was an isocratic mixture of acetonitrile and water (45:55) containing 1% acetic acid. The flow rate of the mobile phase was 1 ml/min.

Standard solutions of OTA and OT $\alpha$  were purchased from Romer Labs Diagnostic GmbH (Tulln, Austria), calibration solutions were prepared in HPLC mobile phase, and calibration curves were prepared in the range of 0.1 to 100 ng/ml.

Twenty microliters of filtered liquid cultures of *A. carbonarius* KB1039 ( $\Delta\text{kusA}$ ) and KB1041 ( $\Delta\text{kusA}\Delta\text{AcOTAnrps}$ ) were also analyzed by HPLC-

**TABLE 2** Summary of HPLC-HRMS retention times and HR mass data of OTA and other metabolites identified in cultures of wild-type and  $\Delta\text{AcOTAnrps}$  strains

Compound	Retention time (min)	Mass (Da)		Error (mDa)	Formula
		Measured	Monoisotopic		
Phe	2.64	165.0782	165.0789	-0.7	C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>
OTB ethyl ester	— <sup>a</sup>	—	399.1682	—	C <sub>22</sub> H <sub>25</sub> NO <sub>6</sub>
OT $\beta$	5.89	222.0516	222.0528	-1.2	C <sub>11</sub> H <sub>10</sub> O <sub>5</sub>
Phe ethyl ester	6.11	193.1093	193.1103	-1.0	C <sub>11</sub> H <sub>15</sub> NO <sub>2</sub>
7-MM	7.61	192.0777	192.0786	-0.9	C <sub>11</sub> H <sub>12</sub> O <sub>3</sub>
OT $\alpha$	8.00	256.0132	256.0138	-0.6	C <sub>11</sub> H <sub>9</sub> ClO <sub>5</sub>
OTC	—	—	431.1136	—	C <sub>22</sub> H <sub>22</sub> ClNO <sub>6</sub>
OTB	18.98	369.1201	369.1212	-1.1	C <sub>20</sub> H <sub>19</sub> NO <sub>6</sub>
OTA	25.27	403.0807	403.0823	-1.6	C <sub>20</sub> H <sub>18</sub> ClNO <sub>6</sub>

<sup>a</sup> —, OTB ethyl ester and OTC were not present in cultures of either wild-type or  $\Delta\text{AcOTAnrps}$  strains.

HRMS for the identification of 7-methylmellein (7-MM), OT $\beta$ , OT $\alpha$ , phenylalanine (Phe), ethyl ester of OTB, OTB, OTC, and OTA. HPLC-HRMS retention times and HR mass data of the metabolites are shown in Table 2. The column was a Kinetex C<sub>18</sub> column (100 mm by 2.10 mm, 2.6  $\mu\text{m}$ ). The mobile phase was a multistep gradient of water (eluent A) and methanol (eluent B), both containing 0.5% acetic acid and 1 mM ammonium acetate. A gradient elution was performed by changing the mobile phase composition as follows. After 3 min at 20% eluent B, the proportion was set at 40% and then linearly increased to 63% in 35 min and kept constant for 11 min. The column was reequilibrated with 20% eluent B for 10 min prior to the successive injection (34). HPLC-HRMS analyses were performed on a benchtop single-stage mass spectrometer (Exactive) equipped with a heated electrospray ion source (HESI II) (Thermo Fisher Scientific, Bremen, Germany), coupled to an Accela HPLC system (Thermo Fisher Scientific, San Jose, CA). The HESI II interface was used in the positive-ion mode, and the scan range was 50.2 to 1,003.0  $m/z$ , with a resolution power of 100,000 FWHM (full width at half maximum). Other settings were as follows: sheath and auxiliary gas flow rates, 30 and 10 arbitrary units, respectively; sweep gas, 0 arbitrary units; capillary temperature,  $300^{\circ}\text{C}$ ; capillary voltage, 4 kV. The Xcalibur software (version 2.1.0, Thermo Fisher Scientific) was used for data acquisition and processing.

**Statistical analysis.** Statistical analysis was performed by using the GraphPad Instat software (Instat, San Diego, CA). Data were subjected to the paired *t* test (two-tailed *P* value) to determine the statistical differences when two groups of data were compared. One-way analysis of variance (ANOVA) with posttest (standard parametric methods) was used when three groups of data were compared.

## RESULTS

**Identification and inactivation of the *AcOTAnrps* gene in *A. carbonarius*.** In the genome of *A. carbonarius* ITEM 5010, we identified a 5,691-bp gene, designated *AcOTAnrps*, encoding a nonribosomal peptide synthetase (PI no. 132610), and located at about 900 nucleotides (nt) upstream of a *pks* gene. The NRPS protein is predicted to contain 1,875 amino acid (aa) residues and is characterized by one module with the typical three core domains and an additional last adenylation domain. The predicted amino acid sequence displays  $\sim 60\%$  identity to the *nrps* gene product of the predicted OTA cluster in *A. niger* (42) and a low similarity (11% identity) to the OTA NRPS in *P. nordicum* (AY534879).

A deletion cassette for *AcOTAnrps* was designed and transformed into strain KB1039 of *A. carbonarius* ITEM 5010, lacking the *kusA* gene, which is required for NHEJ double-strand-break

repair of DNA. Many species of filamentous fungi lacking the NHEJ process show a much higher percentage of targeted gene deletion, and the  $\Delta kusA$  mutant strain was created for this aim in a previous study (Praseuth et al., submitted). Three transformants positive by PCR screening for the integration of the *bar* resistance marker at the *AcOTAnrps* locus were obtained (see Fig. S2 in the supplemental material). One of them was chosen for subsequent analyses and was designated KB1041. A Southern blot hybridization, performed on the genomic DNA of the wild-type and mutant strains digested with BamHI and PstI, confirmed the complete deletion of *AcOTAnrps* gene in KB1041: hybridizing bands of 1.75 kb and 3.0 kb with BamHI and PstI digestions, respectively, indicated the endogenous gene, whereas a homologous integration of the deletion cassette resulted in a band of  $\sim 7.1$  kb when DNA was digested with BamHI and of 6.7 kb when DNA was digested with PstI (Fig. 2C). The RT-PCR analysis shown in Fig. 2B confirmed the lack of expression of the *AcOTAnrps* gene in mutant strain KB1041, when the wild type and the two mutant strains were grown in an OTA permissive medium.

**Production of OTA, OT $\alpha$ , and other secondary metabolites in mutant and wild-type strains of *A. carbonarius*.** Both *A. carbonarius* mutant strains KB1039 ( $\Delta kusA$ ) and KB1041 ( $\Delta kusA \Delta AcOTAnrps$ ) and wild-type strain ITEM 5010 were inoculated into minimal medium and incubated for 7 and 14 days at 25°C in the dark. During the incubation period, no difference in fungal growth, sporulation, and pigment production was observed. In particular, at 7 days the mean biomass weights (triplicate measurements) were  $0.94 \pm 0.13$ ,  $0.82 \pm 0.14$ , and  $0.94 \pm 0.21$  g for the wild-type,  $\Delta kusA$ , and  $\Delta AcOTAnrps$  strains, respectively, and no statistical difference was observed between them ( $P = 0.6295$ ). Similar results were obtained at 14 days ( $P = 0.6815$ ). Cultures were analyzed for the presence of OTA and OT $\alpha$  by HPLC-FLD. Cultures of *A. carbonarius* ITEM 5010 and mutant strains KB1039 and KB1041 were also analyzed for 7-MM, OT $\beta$ , the OTB ethyl ester, Phe, Phe ethyl ester, OT $\alpha$ , OTB, OTC, and OTA by HPLC-HRMS. The simultaneous identification of these metabolites in liquid cultures was possible through use of the Orbitrap mass analyzer, which enables high mass resolution (up to 150,000), high mass accuracy, large dynamic range, and a high mass/charge range (28).

HPLC-FLD analyses showed that OTA and OT $\alpha$  were detected in the cultures of wild-type and KB1039 strains, the latter with the *kusA* gene deleted (Fig. 3A and B). In particular, after 7 days, mean values of OTA and OT $\alpha$  in the wild-type strain culture were 66.7 and 3.2 ng/ml, respectively. In the strain KB1039 lacking in the *kusA* gene, after 7 days, the mean concentrations of OTA and OT $\alpha$  were 54.7 and 2.8 ng/ml, respectively. No statistically significant differences were observed for OTA ( $P = 0.1103$ ) and OT $\alpha$  ( $P = 0.2245$ ) among the wild-type and  $\Delta kusA$  strains. No statistically significant differences were also observed between the two strains after 14 days of growth (OTA, 79.3 and 62.6 ng/ml,  $P = 0.1594$ , and OT $\alpha$ , 5.1 and 4.1 ng/ml,  $P = 0.4663$ , in the wild-type and  $\Delta kusA$  strains, respectively). The HPLC-FLD analyses showed the same metabolite profile for cultures of wild-type strain ITEM 5010 and mutant strain KB1039 ( $\Delta kusA$ ), both producing OTA and OT $\alpha$ . In cultures of mutant strain KB1041, lacking the *kusA* and *AcOTAnrps* genes, neither OTA nor OT $\alpha$  was detected, with a limit of detection (LOD) of 0.1 ng/ml. The lack of OTA production by the mutant strain KB1041 was also confirmed when liquid cultures were purified and concentrated with IMA columns,

which permitted the lowering of the LOD from 0.1 ng/ml to 0.005 ng/ml (Fig. 3C). The absence of OTA and OT $\alpha$  in liquid cultures of KB1041 was further confirmed by HPLC-HRMS. Comparison of HPLC-HRMS metabolite profiles between mutant strains KB1039 and KB1041 in triplicate experiments showed the following major differences among these strains: OTA, OTB, and OT $\alpha$  which occurred in relevant amounts in the culture of mutant strain KB1039, lacking the *kusA* gene, were not present in mutant strain KB1041, lacking the *kusA* and *AcOTAnrps* genes. Other pieces of evidence from HRMS analysis were a clear and significant increase of OT $\beta$  ( $P = 0.0018$ ) in the culture of mutant strain KB1041 compared to mutant strain KB1039 and the presence of 7-MM, Phe, and ethyl ester of Phe in cultures of both strains. Neither ethyl ester of OTA (OTC), nor ethyl ester of OTB, a hypothetical intermediary before OTB formation, was detected in either cultures (Fig. 4).

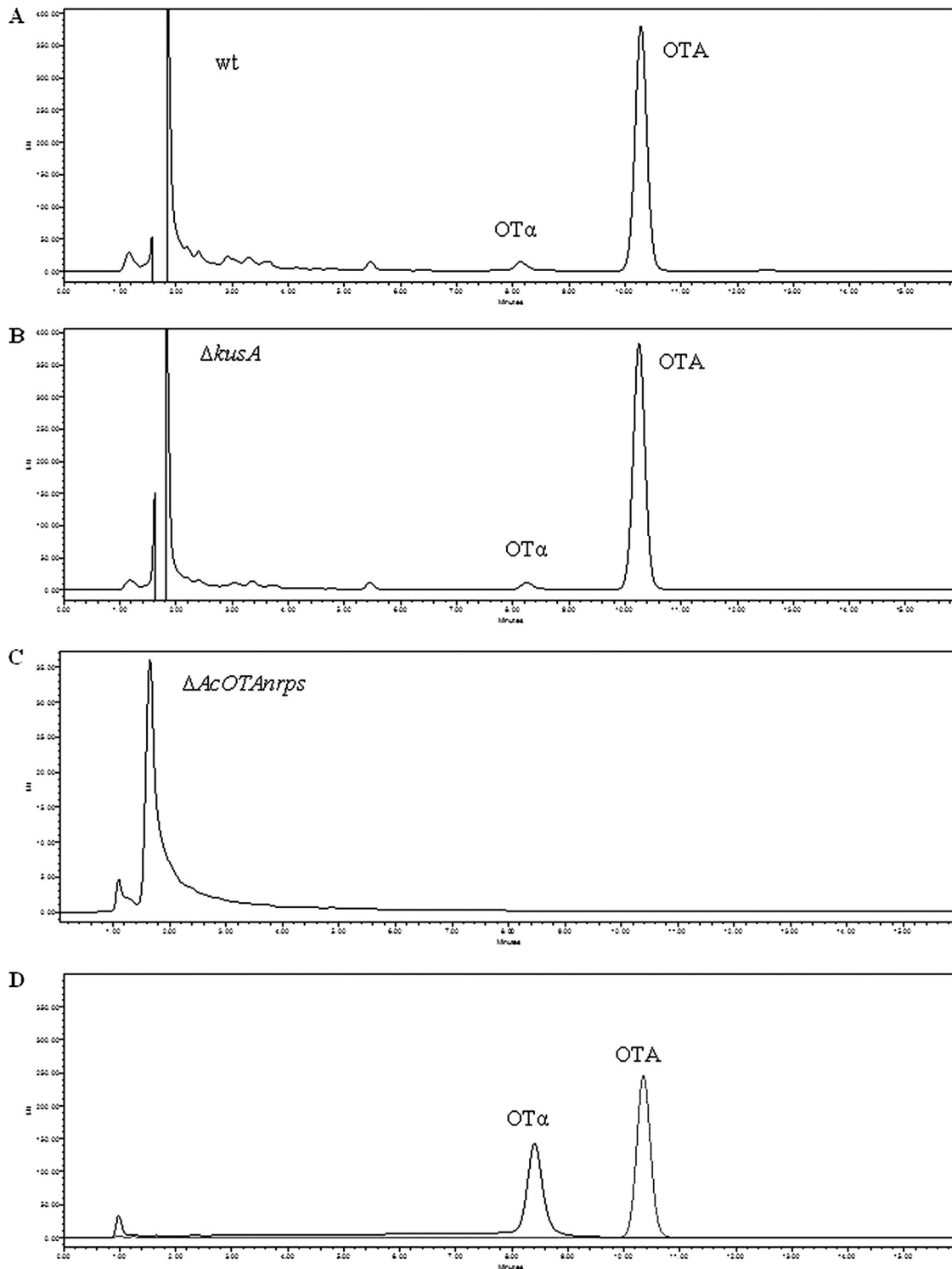
**Kinetics of OTA degradation by wild-type and  $\Delta AcOTAnrps$  mutant strains of *A. carbonarius*.** The *A. carbonarius* strain lacking in functional *AcOTAnrps* was tested for its ability to degrade OTA. To examine the kinetics of OTA degradation, toxin was added in 100 ml of MM culture (3  $\mu$ M) prior to inoculum of the fungus, and the presence of OTA and OT $\alpha$  was monitored for 25 days in a 1-ml sample taken every day.

As shown in Fig. 5, the measured concentrations of OTA in control medium (no fungus) and at time zero of medium inoculated with fungus were the same (about 3  $\mu$ M), and no OT $\alpha$  was detected in either culture. The initial amount of OTA measured in the medium was degraded almost completely (>99%) after 2 days of incubation. As a result of OTA degradation, OT $\alpha$  appeared in the medium already 1 day after the addition of OTA. The formation of OT $\alpha$  could be the result of OTA hydrolysis produced by the mutant strain. In particular, the OT $\alpha$  concentration rose from 0.09  $\mu$ M after 1 day to a maximum of about 0.8  $\mu$ M (27% of the OTA concentration measured at time zero) already after 7 days and was constant until the end of the experiment. In the uninoculated control, the concentration of OTA remained constant over the time course.

## DISCUSSION

Ochratoxin A is a chlorinated polyketide molecule containing the amino acid phenylalanine. According to the molecular structure of OTA, several enzymatic activities are required for the biosynthetic process, such as a polyketide synthase for the synthesis of the polyketide dihydroisocoumarin, a chlorinating enzyme, and also a peptide synthetase, which is necessary to link the phenylalanine to the dihydroisocoumarin ring. However, the order in which these enzymes act in the biosynthetic pathway has, until this study, not yet been clarified. Most studies of OTA biosynthetic genes have been focused on *pks* genes, which are involved in the first steps of the pathway. These *pks* genes have been shown experimentally to have a role in OTA biosynthesis of different fungal species of the genera *Aspergillus* and *Penicillium* (24). In *Penicillium*, other OTA production genes have been identified and investigated; an *nrrps* gene, encoding a nonribosomal peptide synthetase, was identified to be responsible for the formation of the peptide bond between the polyketide dihydroisocoumarin and the amino acid phenylalanine (31).

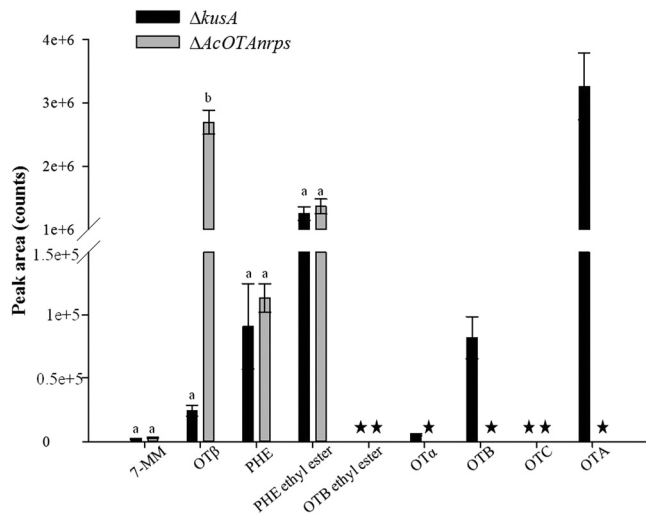
Nonribosomal peptide synthetases are multimodular enzymes present in large numbers in the genomes of filamentous fungi. Both PKs and NRPSs constitute a class of multifunctional pro-



**FIG 3** HPLC-FLD chromatograms of OTA and OT $\alpha$ . (A) Culture medium of wild-type *A. carbonarius*; (B) culture medium of  $\Delta kusA$  mutant KB1039; (C) culture medium of  $\Delta AcOTAnrps$  mutant KB1041 purified by IMA column before HPLC-FLD analysis; (D) OTA and OT $\alpha$  standards. Retention times: OTA, 10.35 min; OT $\alpha$ , 8.41 min.

teins involved in the synthesis of a multitude of secondary metabolites with a wide range of biological activities. A great number of natural products of microbial origin are biosynthetically derived from amino acids and carboxylic acid moieties. The synthesis of

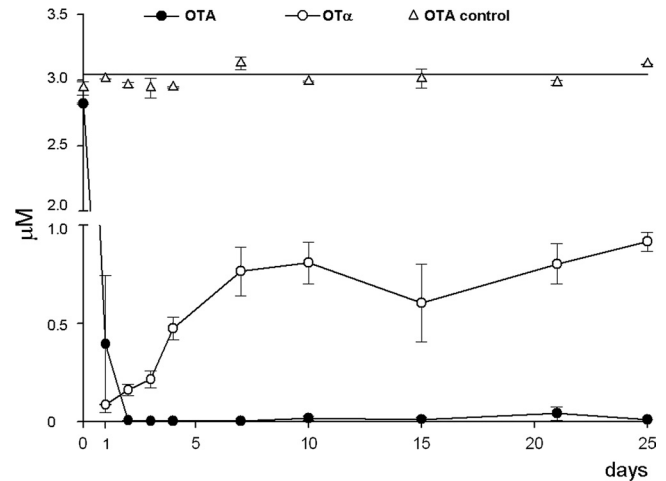
these mixed peptide-polyketide natural products may be catalyzed by a hybrid NRPS-PKS system involving direct interactions between NRPS and PKS modules, or the peptide-polyketide backbone is assembled without a direct functional hybridization be-



**FIG 4** Metabolites supposed to be involved in OTA biosynthesis and identified by HPLC-HRMS in cultures of  $\Delta kusA$  (KB1039) and  $\Delta AcOTAnrps$  (KB1041) strains incubated in triplicate for 7 days. \*, not detected. Values are means  $\pm$  standard errors. Different letters indicate statistical differences within pairs of data ( $P < 0.005$ ).

tween NRPS and PKS proteins, which act individually (16). In particular, NRPSs have been proposed to operate via a thiotemplate mechanism in a manner independent of the ribosome. They exhibit a modular structure, with one module being a semiautonomous unit that recognizes, activates, and modifies a single residue of the final peptide by means of three typical domains: (i) the adenylation domain (A), which is responsible for substrate specificity and activation; (ii) the peptidyl carrier domain (P), which covalently binds the substrate to the enzymes via a thioester linkage; and (iii) the condensation domain (C), which catalyzes peptide bond formation (20, 50, 56). Our analyses of the recently sequenced *A. carbonarius* genome revealed the presence of about 13 complete *nrps* genes, one of which was identified as being in the OTA cluster based on homology to the predicted OTA biosynthetic cluster of *A. niger* (42). The gene we designated *AcOTAnrps* is about 900 nt upstream of a *pks* gene, and they are transcribed in the same direction, differently from *P. nordicum*, in which the OTA *pks* and *nrps* genes are transcribed in the opposite direction (31). *AcOTAnrps* encodes a protein (PI no. 132610) with 1,876 aa, characterized by one module with the typical three core domains and an additional last adenylation domain (see Fig. S3 in the supplemental material). Monomodular NRPS-related enzymes involved in secondary metabolites have so far not been well characterized (55), but the complete module identified in the *AcOTAnrps* product would be sufficient for ligation of the isocoumarin group to the amino acid phenylalanine through the carboxylic group in the OTA molecule. We confirmed this hypothesis by gene inactivation. The resulting strain of *A. carbonarius* lacking the *nrps* gene was incapable of OTA production, proving that the *AcOTAnrps* gene is involved in the biosynthetic pathway of OTA.

This is the first report about the identification in *Aspergillus* species of an *nrps* gene involved in OTA biosynthesis. Moreover, our attention was focused on the gene encoding a peptide synthetase with the aim of elucidating the order of the reactions in the biosynthetic pathway, which is still not completely defined. For this reason, the presence of OT $\alpha$  also was monitored during the



**FIG 5** Kinetics of OTA degradation and OT $\alpha$  formation by the  $\Delta AcOTAnrps$  (KB1041) mutant during 25 days of incubation in minimal medium spiked with 3  $\mu$ M OTA. The concentration of OTA remained unchanged in control medium over the entire duration of the experiments. Values are means  $\pm$  standard errors.

analysis of OTA production by the mutant strain. Ochratoxin  $\alpha$  is the chlorinated isocoumaric derivative of OTA not linked to phenylalanine, which is identified naturally and usually detected in the cultures of OTA-producing species. We observed its presence parallel to the production of OTA in cultures of the *A. carbonarius* wild-type and  $\Delta kusA$  strains analyzed after 7 and 14 days of incubation. The role of OT $\alpha$  in the biosynthetic pathway was not clear. According to the predominant hypothesis of Huff and Hamilton (29), it is derived from the chlorination of OT $\beta$  by a chloroperoxidase and then linked to the ethyl ester of phenylalanine via a synthetase to generate OTC, which is finally deesterified to produce OTA. Harris and Mantle (27) suggested that the conversion of OT $\alpha$  to OTA is the main biosynthetic pathway, with the chlorinating step being intermediate and prior to ligation of the polyketide to the phenylalanine molecule. However, their feeding experiments have shown that chlorination of OT $\beta$  also can give rise to OTA, leading to an alternative pathway that entails the conversion of OT $\beta$  to OTB and then the chlorination to OTA, with the chlorinating step as the last reaction and without the direct involvement of OT $\alpha$ , even though the latter was demonstrated to occur naturally. One of our initial hypotheses was that the inactivation of the *nrps* gene, which encodes the peptide synthetase responsible of the ligation of phenylalanine moiety to the polyketide, could aid in clarifying this question. In the cultures of the  $\Delta AcOTAnrps$  strain, which was unable to produce OTA, the absence of OT $\alpha$  was observed, contrary to the hypothesis involving chlorination of the intermediate OT $\alpha$ ; in this hypothesis, accumulation of OT $\alpha$  would result from a block at the phenylalanine addition step. The absence of OTA and OT $\alpha$  in the cultures of the mutant strain was also confirmed by HPLC-HRMS analysis. Other related metabolites were analyzed by HRMS, and among them, the ethyl esters of OTA (OTC) and of OTB were not detected in the cultures of both producing and mutant strains (Fig. 4). This result suggests that probably phenylalanine and not its esterified form is involved in the bond with the carboxy group of OT $\beta$ . The hypothesis of esterification of phenylalanine in the biosynthesis pathway was proposed by Huff and Hamilton (29) for its



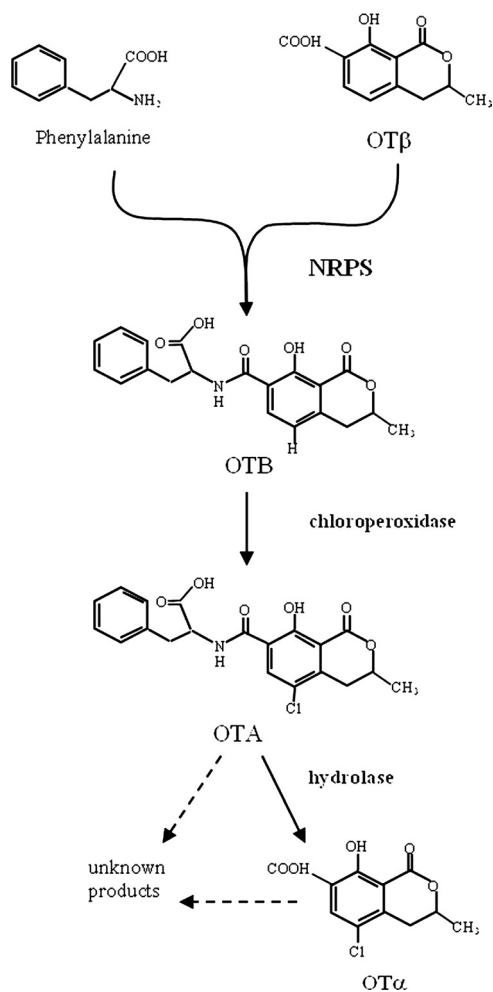


FIG 6 New schematic representation of the last steps of the OTA biosynthetic pathway as proposed in this work.

predicted protective role of the phenylalanine carboxy group in the binding reaction leading to OTC. However, in their subsequent study, Harris and Mantle (27) found no evidence for the intermediate role of OTC.

Moreover, from our HRMS analysis, the absence of OTB and, at the same time, a statistically significant ( $P = 0.0018$ ) increase in OTβ, accumulating in the medium as a precursor of OTB as a consequence of *nrps* inactivation, confirm the hypothesis that the step of ligation of phenylalanine to polyketide catalyzed by an NRPS most occurs prior to the chlorination step (Fig. 6). Furthermore, this finding is corroborated by the studies of Geisen et al. (25) on the expression kinetics of the known OTA-related genes in *P. nordicum*, in correlation with the OTA biosynthesis. They observed a consecutive occurrence of expression optima of the various genes, which indicated the enzymatic step of NRPS occurred before the chlorination step, with the latter step being the last reaction just before OTA excretion. According to these authors, this putative order of reactions agrees with a possible self-protection mechanism of the producing organism from the toxic OTA at the completion of biosynthesis.

One of the historical arguments against this hypothesis of the biosynthetic pathway has been the difficulty to explain the natural

occurrence of OTα in fungal cultures if it is not directly involved in biosynthesis. However, several reports have now been published about the presence of OTα as a product of OTA biodegradation by different microorganisms, among which are various *Aspergillus* species (2, 8, 54). The amide bond between OTα and phenylalanine in OTA is hydrolyzed by several different enzymes, including a carboxypeptidase A (15), lipase (51), commercial proteases, and as recently determined, a metalloprotease isolated in *A. niger* (3).

In the light of the hypothesis we present in this work regarding the OTA biosynthetic pathway, the presence of OTα in the OTA-producing *A. carbonarius* strain culture is most likely due to the biodegradation activity of the fungus and is independent of OTA biosynthesis. This is also confirmed by the fact that the mutant strain, lacking the *AcOTAnrps* gene and therefore unable to produce the mycotoxin, retains the capability to degrade OTA. Ochratoxin A was quickly and completely degraded, with only 14% remaining in culture after only 1 day and with less than 1% remaining after 2 days of incubation with exogenous OTA. OTα was detected after only 1 day, and the amount of OTα that had accumulated in culture at the end of incubation was equivalent to 27% of the initial amount of OTA, suggesting that OTA could be degraded to other metabolites in addition to OTα (23) or that OTα could be degraded further. The degradation process appeared faster and more significant than those observed in other studies in which the kinetics of nonochratoxigenic *Aspergillus* species were analyzed (2, 8). From the results of this experiment, it was not possible to have an indication of whether the hydrolysis activity occurred in the medium due to an enzyme excreted out of the cell or if externally applied OTA was absorbed into the cell where the degradation process would have taken place. Previous studies have indicated that OTA is a substrate for different kinds of transporters facilitating not only the efflux of compounds but also cellular uptake (49). Actually, contradictory findings have been reported about the excretion of OTA-degrading enzymes by different fungal species. Abrunhosa and Venancio (3) purified the metalloprotease responsible for OTA hydrolysis from the culture medium of *A. niger*, after separation of the fungal cell, whereas Peteri et al. (45) reported that the enzyme responsible of OTA degradation in *Phaffia rhodozyma* was not excreted, because the simple fermented broth was unable to degrade OTA. A specific OTA degradation activity of the producing strain could be supposed as the strategy of a self-protection mechanism. Also in the biosynthesis of trichothecenes by *Fusaria*, a mechanism of self-defense for producers was found, related to the acetylation of the trichothecene ring owing to the *tri101* gene of the biosynthetic cluster (33). Additional investigation of the OTA biosynthetic cluster of *A. carbonarius* would be worthwhile, with the aim of finding a specific degradation gene product for a better explanation of mycotoxin biosynthesis and in view of its potential application.

## ACKNOWLEDGMENTS

This work was partially supported by the Italian Ministry of Economy and Finance (MEF) Project, CISIA-Conoscenze Integrate per la Sostenibilità e l'Innovazione del *made in Italy* Agroalimentare, and by the private-public initiative Molecular Biodiversity Laboratory, partially funded by the Italian Ministry of University and Research (MIUR), Fondo FAR Legge 297/1999 Art. 12/lab project grant DM19410. The genome sequencing of *Aspergillus carbonarius* was conducted by the U.S. Department of Energy

Joint Genome Institute and supported by the Office of Science of the U.S. Department of Energy under contract no. DE-AC02-05CH11231. We appreciate the DOE Office of the Biomass Program support for the research conducted at the Pacific Northwest National Laboratory.

We thank Filomena Epifani, Giuseppe Panzarini, and Roberto Schena for valuable technical assistance.

## REFERENCES

- Abouzed MM, et al. 2002. Ochratoxin A concentrations in food and feed from a region with Balkan endemic nephropathy. *Food Addit. Contam.* 19:755–764.
- Abrunhosa L, Serra R, Venancio A. 2002. Biodegradation of ochratoxin A by fungi isolate from grapes. *J. Agric. Food Chem.* 50:7493–7496.
- Abrunhosa L, Venancio EA. 2007. Isolation and purification of an enzyme hydrolyzing ochratoxin A from *Aspergillus niger*. *Biotechnol. Lett.* 29:1909–1914.
- Andersen MR, et al. 2011. Comparative genomics of citric-acid-producing *Aspergillus niger* ATCC 1015 versus enzyme-producing CBS 513.88. *Genome Res.* 21:885–897.
- Atoui A, Dao HP, Mathieu F, Lebrihi A. 2006. Amplification and diversity analysis of ketosynthase domains of putative polyketide synthase genes in *Aspergillus ochraceus* and *Aspergillus carbonarius* producers of ochratoxin A. *Mol. Nutr. Food Res.* 50:488–493.
- Bacha N, Atoui A, Mathieu F, Liboz T, Lebrihi A. 2009. *Aspergillus westerdijkiae* polyketide synthase gene “*aoks1*” is involved in the biosynthesis of ochratoxin A. *Fungal Genet. Biol.* 46:77–84.
- Battilani P, Magan N, Logrieco A. 2006. European research on ochratoxin A in grapes and wine. *Int. J. Food Microbiol.* 111(Suppl 1):S2–S4.
- Bejaoui H, Mathieu F, Taillandier P, Lebrihi A. 2006. Biodegradation of ochratoxin A by *Aspergillus* section *Nigri* species isolated from French grapes: a potential means of ochratoxin A decontamination in grape juice and musts. *FEMS Microbiol. Lett.* 255:203–208.
- Belli N, et al. 2004. Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. *J. Sci. Food Agric.* 84:541–546.
- Botton A, et al. 2008. A cDNA-AFLP approach to study ochratoxin A production in *Aspergillus carbonarius*. *Int. J. Food Microbiol.* 127:105–115.
- Chiang YM, Oakley BR, Keller NP, Wang CC. 2010. Unraveling polyketide synthesis in members of the genus *Aspergillus*. *Appl. Microbiol. Biotechnol.* 86:1719–1736.
- Cramer RA, et al. 2006. Phylogenomic analysis of non-ribosomal peptide synthetase in the genus *Aspergillus*. *Gene* 383:24–32.
- Crespo-Sempere A, Gonzales-Candela L, Martinez-Culebras PV. 2010. Genes differentially expressed by *Aspergillus carbonarius* strains under ochratoxin A producing conditions. *Int. J. Food Microbiol.* 142:170–179.
- Crespo-Sempere A, Gil JV, Martinez-Culebras PV. 2011. Proteome analysis of the fungus *Aspergillus carbonarius* under ochratoxin A producing conditions. *Int. J. Food Microbiol.* 147:162–169.
- Deberghes P, et al. 1995. Detoxification of ochratoxin A, a food contaminant: prevention of growth of *Aspergillus ochraceus* and its production of ochratoxin A. *Mycotox. Res.* 11:37–47.
- Du L, Sanchez C, Shen B. 2001. Hybrid peptide-polyketide natural products: biosynthesis and prospects toward engineering novel molecules. *Metab. Eng.* 3:78–95.
- EFSA. 2006. Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to ochratoxin A in food. *EFSA J.* 365:1–56.
- EFSA Panel on Contaminants in the Food Chain. 2010. Statement on recent scientific information on the toxicity of ochratoxin A. *EFSA J.* 8:1626. doi:10.2933/j.efsa.2010.1626.
- FAO/WHO. 2001. Safety evaluation of certain mycotoxins in food. Fifty-Sixth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)—WHO Food Additives series 47. FAO food and nutrition paper 74. Food and Agriculture Organization, World Health Organization, Geneva, Switzerland.
- Finking R, Marahiel MA. 2004. Biosynthesis of non ribosomal peptides. *Annu. Rev. Microbiol.* 58:453–488.
- Frisvad JC, et al. 2011. Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. *PLoS One* 6:e23496. doi:10.1371/journal.pone.0023496.
- Gallo A, et al. 2009. Characterisation of a *pks* gene which is expressed during ochratoxin A production by *Aspergillus carbonarius*. *Int. J. Food Microbiol.* 129:8–15.
- Galtier P, Alvinaire M. 1976. In vitro transformation of ochratoxin A by animal microbial floras. *Ann. Rech. Vet.* 7:91–98.
- Geisen R, Schmidt-Heydt M. 2009. Physiological and molecular aspects of ochratoxin A biosynthesis, p 353–376. In Anke T, Weber D (ed), *Physiology and genetics*. The Mycota XV. Springer-Verlag, Heidelberg, Germany.
- Geisen R, Schmidt-Heydt M, Karolewicz A. 2006. A gene cluster of the ochratoxin A biosynthetic genes in *Penicillium*. *Mycotox. Res.* 22:134–141.
- Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61:1323–1330.
- Harris JP, Mantle PG. 2001. Biosynthesis of ochratoxins by *Aspergillus ochraceus*. *Phytochemistry* 558:709–716.
- Hu Q, et al. 2005. The Orbitrap: a new mass spectrometer. *J. Mass Spectrom.* 40:430–443.
- Huff WE, Hamilton PB. 1979. Mycotoxins—their biosynthesis in fungi: ochratoxins—metabolites of combined pathways. *J. Food Prot.* 42:815–820.
- IARC. 1993. Some naturally occurring substances, food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC monographs on the evaluation of carcinogenic risks to humans, vol. 56. IARC, Lyon, France.
- Karolewicz A, Geisen R. 2005. Cloning a part of the ochratoxin A biosynthetic gene cluster of *Penicillium nordicum* and characterization of the ochratoxin polyketide synthase gene. *Syst. Appl. Microbiol.* 28:588–595.
- Keller NP, Turner G, Bennet JW. 2005. Fungal secondary metabolism—from biochemistry to genomics. *Nat. Rev. Microbiol.* 3:937–947.
- Kimura M, Shingu Y, Yoneyama K, Yamaguchi I. 1998. Features of *Tri101*, the trichothecene 3-O-acetyltransferase gene, related to the self defense mechanism in *Fusarium graminearum*. *Biosci. Biotechnol. Biochem.* 62:1033–1036.
- Lattanzio VMT, Solfrizzo M, Visconti A. 2007. Simultaneous determination of aflatoxins, ochratoxin A and *Fusarium* toxins in maize by liquid chromatography/tandem mass spectrometry after multitoxin immunoaffinity cleanup. *Rapid Commun. Mass Spectrom.* 22:3253–3261.
- Lund F, Frisvad JC. 2003. *Penicillium verrucosum* in cereals indicates production of ochratoxin A. *J. Appl. Microbiol.* 95:1117–1123.
- Magnoli C, et al. 2007. Ochratoxin A and *Aspergillus* section *Nigri* in peanut seeds at different months of storage in Cordoba, Argentina. *Int. J. Food Microbiol.* 119:213–218.
- Maniatis T, Fritsch EF, Sambrook J. 1982. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Martinez-Culebras PV, et al. 2009. Molecular characterization of the black *Aspergillus* isolates responsible for ochratoxin A contamination in grapes and wine in relation to taxonomy of *Aspergillus* section *Nigri*. *Int. J. Food Microbiol.* 132:33–41.
- Moss MO. 1996. Mode of formation of ochratoxin A. *Food Addit. Contam.* 13:5–9.
- Moss MO. 1998. Recent studies of mycotoxins. *Symp. Ser. Soc. Appl. Microbiol.* 84:62S–76S.
- O’Callaghan J, Caddick MX, Dobson ADW. 2003. A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. *Microbiology* 149:3485–3491.
- Pel HJ, et al. 2007. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol.* 25:221–231.
- Perrone G, et al. 2006. Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. *Appl. Environ. Microbiol.* 72:680–685.
- Perrone G, et al. 2007. Biodiversity of *Aspergillus* species in some important agricultural products. *Stud. Mycol.* 59:53–66.
- Peteri Z, Téren J, Vagvölgyi C, Varga J. 2007. Ochratoxin degradation and adsorption caused by astaxanthin-producing yeasts. *Food Microbiol.* 24:205–210.
- Petzinger E, Ziegler K. 2000. Ochratoxin A from a toxicological perspective. *J. Vet. Pharmacol. Ther.* 23:91–98.
- Pfohl-Leschkowicz A, Manderville RA. 2007. Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. *Mol. Nutr. Food Res.* 51:61–99.

48. Sanchez-Hervas M, Gil JV, Bisbal F, Ramon D, Martinez-Culebras PV. 2008. Mycobiota and mycotoxin producing fungi from cocoa beans. *Int. J. Food Microbiol.* **125**:336–340.
49. Schrickx J, Lekatarau Y, Fink-Gremmels J. 2006. Ochratoxin A secretion by ATP-dependent transporters in Caco-2 cells. *Arch. Toxicol.* **80**:243–249.
50. Schwarzer D, Finking R, Marahiel MA. 2003. Nonribosomal peptides: from genes to products. *Nat. Prod. Rep.* **20**:275–287.
51. Stander MA, Bornscheuer UT, Henke E, Steyn PS. 2000. Screening of commercial hydrolases for the degradation of ochratoxin A. *J. Agric. Food Chem.* **48**:5736–5739.
52. Taniwaki MH, Pitt JI, Teixeira AA, Iamanaka BT. 2003. The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. *Int. J. Food Microbiol.* **82**:173–179.
53. Van der Merwe KJ, Steyn PS, Fourie L. 1965. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh. *Nature* **205**:1112–1113.
54. Varga J, Rigo K, Teren J. 2000. Degradation of ochratoxin A by *Aspergillus* species. *Int. J. Food Microbiol.* **59**:1–7.
55. von Dohren H. 2009. A survey of nonribosomal peptide synthetase (NRPS) genes in *Aspergillus nidulans*. *Fungal Genet. Biol.* **46**:S45–S52.
56. Weber T, Marahiel MA. 2001. Exploring the domain structure of modular nonribosomal peptide synthetase. *Structure* **9**:R3–R9.
57. Xiao H, Marquard RR, Frohlich AA, Ling YZ. 1995. Synthesis and structural elucidation of analogues of ochratoxin A. *J. Agric. Food Chem.* **43**:524–530.
58. Yu JH, et al. 2004. Double joint PCR: a PCR based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet. Biol.* **41**:973–981.