DEGRADATION OF OCHRATOXIN A BY *Brevibacterium* spp

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ABSTRACT

The ability to degrade Ochratoxin A (OTA) was studied in different bacteria with a well-known capacity to transform aromatic compounds. Strains belonging to *Rhodococcus*, *Pseudomonas* and *Brevibacterium* genera were grown in liquid synthetic culture medium containing OTA. *Brevibacterium* spp strains showed 100% degradation of OTA. Ochratoxin α (OTα) was detected and identified by high-performance liquid chromatography-mass spectrometry (HPLC-MS) as degradation product in the cell-free supernatants. We demonstrated for the first time the degradation of OTA by bacteria belonging to the food chain, which is of public concern for food and environmental safety.

Keywords

ochratoxin A; ochratoxin α; *Brevibacterium*; degradation; mycotoxin
INTRODUCTION

Ochratoxin A (OTA), an isocumarin derivative linked via the carboxyl group to L-β-phenylalanine, is a mycotoxin produced by several species of fungi belonging principally to the genera *Aspergillus* and *Penicillium*. These fungi are capable to grow under a wide range of conditions, including moisture, pH, and temperature, and on a variety of foods, such as cereals, coffee, fruits, cocoa, nuts, spices, meat, milk, wine and beer (1, 2). Cereals and cereal derivatives remain the major contributors to OTA human and animal exposure (3).

OTA is suspected to be nephrotoxic, teratogenic, hepatotoxic and carcinogenic (4) and has been detected in human blood after the consumption of contaminated foods (5). To protect consumer from the risk of exposure to this mycotoxin, reliable methods are needed for reducing the levels of OTA in foodstuff. In this regard, it has been shown that decaffeination with solvents significantly reduced OTA levels in coffee (6), and it has also been suggested that contaminated grains can be detoxified of OTA by ozone treatment (7). Likewise, several studies have focused on the reduction of OTA in musts and wines in the winery, and different decontamination procedures based on either physical, chemical, or biological removal have been proposed (8, 9, 10, 11).

With regard to the biological degradation of OTA, enzymes with carboxypeptidase A activity (CPA), such as that obtained from bovine pancreas, have been described. These enzymes can hydrolyze the amidic bond in the OTA molecule with the production of L-phenylalanine (PHE) and ochratoxin α (OTα) (Figure1), which is 500 times less toxic than OTA (12). Likewise, degradation of OTA by *Phenylobacterium immobile* (13), by *Acinetobacter calcoaceticus* (14) and by *Aspergillus* species (15, 16) by a mechanism of action similar to that of CPA, have been reported, although in the later case OTα was produced. More recently, a protein produced by the fungus *Aspergillus niger* able to
hydrolyze OTA to PHE and OTα has been isolated (17). Even more, this mechanism has
been also implicated in the capability of Trichosporon mycotoxinivorans to degrade OTA
(18). Therefore, biological degradation of toxins is a very promising approach for
decontamination of foodstuffs, as use of chemical or physical tools in food may also
remove, along with the mycotoxin, other organoleptic important substances and nutrients.

On the other hand, soil bacteria, such as Pseudomonas spp. and actinobacteria, are able
to transform a wide range of aromatic compounds and, thus, they are considered to play a
crucial role in the biodegradation of toxic pollutants in soil (19, 20, 21). For instances,
among actinobacteria, Rhodococcus strains are able to degrade a large variety of organic
compounds. In fact, over 200 genes that encode for oxygenases and more than 30
metabolic pathways involved in the catabolism of aromatic compounds have been
identified in the complete genome sequence of Rhodococcus jostii RHA1 (22). Moreover,
it has recently been published that cell extracts of Rhodococcus erythropolis can degrade
aflatoxin B1, a mycotoxin similar to OTA that also possess an aromatic ring in its
structure (23). Furthermore, some species from another actinobacteria genus,
Brevibacterium, that are usually found in milk and cheese curd, such as B. casei, B.
iodinum, and B. linens (which usual habitat is the exterior of surface-ripened cheeses such
as Limburger, Romadour, Munster, Tilsiter, Appenzeller, Gruyère, Brick and others) have
also been isolated from soil. In addition to their versatility, another important
characteristic of the actinobacteria from the genus Brevibacterium is their extracellular
proteinase production. Intracellular and extracellular cell-wall associated proteinases have
been reported for B. linens, but most studies has focused on the later largely because of
their high activity and importance respect to cheese ripening (24, 25).

As mentioned above, given its potential health hazard, presence of OTA in foods is of
considerable public concern for the food industry and regulatory agencies and,
consequently, there is a growing demand from control authorities to reduce the allowable
limits of OTA in foods and beverages. Therefore, and based on the aforementioned characteristics of some microorganisms, in this study we screened bacteria with the ability to degrade aromatic compounds, such as *Pseudomonas putida* and several actinobacteria from the genera *Rhodococcus* and *Brevibacterium*, for the ability to degrade OTA.

**MATERIALS AND METHODS**

**Microorganisms and growth conditions.** Cultures of the strains *Rhodococcus erythropolis* CECT 3008, *Rhodococcus erythropolis* IGTS8, *Pseudomonas putida* DSMZ 291, *P. putida* KT2442 and seven strains of *Brevibacterium* species were screened for their ability to degrade OTA.

*Rhodococcus erythropolis* CECT 3008 (DSMZ 43060) was purchased from the Spanish Type Culture Collection (CECT). *Pseudomonas putida* DSM 291<sup>T</sup> and the six *Brevibacterium* strains included in the study (*Brevibacterium epidermidis* DSM 20660<sup>T</sup>, *Brevibacterium iodinum* DSM 20626<sup>T</sup>, *Brevibacterium linens* DSM 20425<sup>T</sup>, *Brevibacterium casei* DSM 20657<sup>T</sup>, *B. casei* DSM 9657, *B. casei* DSM 20658) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). A *B. casei* strain, RM101, molecularly identified by 16S rDNA sequencing, was isolated at the Instituto de Fermentaciones Industriales IFI-CSIC. *Rhodococcus erythropolis* IGTS8 and *Pseudomonas putida* KT2442 strains were kindly provided by Dr. Eduardo Diaz, from the Centro de Investigaciones Biológicas, CSIC, Spain.

All bacteria assayed were routinely grown in Luria-Bertani broth (LB) supplemented with 0.5% glucose and incubated at 30 °C under aerobic conditions. For the OTA degradation assay, bacteria were grown in a basal salts medium (BSM) that contained 0.2% glycerol, 4 g of NaH<sub>2</sub>PO<sub>4</sub>-H<sub>2</sub>O, 4 g of K<sub>2</sub>HP<sub>0</sub>₄-3H<sub>2</sub>O, 2 g of NH₄Cl, 0.2 g of MgCl<sub>2</sub>-6H<sub>2</sub>O, 0.001 g of CaCl<sub>2</sub>-2H<sub>2</sub>O, and 0.001 g of FeCl<sub>3</sub>-6H<sub>2</sub>O (26). Glycerol was omitted.
from experiments designed to determine the potential use of OTA as carbon source by the bacteria assayed.

**Standards.** OTA was purchased from Sigma (Sigma Chemical Co, Poole, Dorset, UK) and suspended in 99% methanol under sterile conditions to make a stock solution of 500 mg/L. A standard solution of OTα (11.9 mg/L) was purchased from LGC Standards (Germany) and diluted with acetonitrile (1:1, v/v) to make a working solution of 5.9 mg/L.

Phenylalanine was purchased from VWR (Milano, Italy) and suspended in HCl 0,1 M to make a stock solution of 1 g/L. The standard solution was made in 75% methanol and contained 15 mg/L of PHE. Norvaline was purchased from Sigma (Sigma Chemical Co, Poole, Dorset, UK) and suspended in HCl 0,1 M to make a stock solution of 500 mg/L.

**OTA degradation assay.** Actinobacteria (*Rhodococcus erythropolis* CECT 3008, *Rhodococcus erythropolis* IGTS8, and *Brevibacterium casei* RM101) and *Pseudomonas* spp. (*P. putida* DSMZ 291T and *P. putida* KT2442) strains were grown in 25 mL of BSM containing OTA (about 10 μg/L) under aerobic conditions at 30°C until the late exponential growth phase, time at which supernatants were collected for the analysis of OTA by high-performance liquid chromatography (HPLC). To confirm OTA degradation by *Brevibacterium* spp. strains, the bacterial strains were grown in BSM containing a 4-fold increase on the OTA concentration (40 μg/L) under aerobical conditions on a rotary shaker at 150 rpm for 10 days. To determine the ability of *Brevibacterium* spp. (*B. casei* RM101 and *B. linens* DSM 20425T strains) to degrade OTA at higher concentrations (40 mg/L, 1000 times greater than previously assayed) and to assess the use of OTA as carbon source, bacteria were grown in BSM with or without glycerol (0.2%) and OTA (40 mg/L), and OTA degradation was verified after 10 days of incubation at 30°C. In addition, to
determine the timecourse of OTA degradation and to quantify OTα production, samples from *B. casei* RM101 strain grown in BSM containing OTA (25 mg/L), with or without glycerol (0.2%), were collected twice daily and analyzed by HPLC. In all these degradation assays, culture supernatants were separated by centrifugation at 3000 x g for 10 min at 4º C and further analyzed by HPLC. The *Brevibacterium* spp. cell pellets were stored at -80 ºC for subsequent analysis. BSM controls with OTA and without bacteria were always prepared.

**OTA, OTα and Phenylalanine quantification by HPLC.** Concentration of OTA in supernatants, pellets and pellet’s washing solutions was quantified as described (27) using a Hewlett–Packard I model 1100 series HPLC instrument (Hewlett–Packard, Palo Alto, CA), equipped with a degasser, quaternary pump, auto sampler, Uv/vis (DAD) and fluorescence (FLD) detector and a 200 mm–4,6 mm Alltima C18 (5 µm) column. The mobile phase was: solvent (A), acetonitrile; solvent (B), water (HPLC grade)/acetonitrile/glacial acetic acid (89:10:1 by vol.); solvent A:B=37:63 (v/v), isocratic method; flow, 1.3 mL/min; analysis temperature, 30º C; analysis time, 20 min; FLD detector (λ<sub>ex</sub>=330 nm, λ<sub>em</sub>=460 nm); injection volume, 100 µL. The limit of detection for OTA in the aforementioned conditions is 0.02 µg/L. OTA standard was injected at two different concentrations: 20 and 50 µg/L; OTα standard was injected at a concentration of 5.9 mg/L. Samples were appropriated diluted with solvent A:B before the analysis HPLC.

The *Brevibacterium* spp. bacterial pellets were resuspended twice in 2 ml of absolute methanol for 1 h to extract the OTA. After centrifugation at 3,000 x g for 15 min at 20º C, the methanolic supernatants were separated, collected in 5 ml vials and evaporated to dryness with a stream of dry nitrogen gas. The dry residues were reconstituted with the mobile phase immediately before analysis for the determination of OTA concentration.
Concentration of PHE in supernatants was quantified as described (28) by using the same instrument and column mentioned for OTA analysis. Briefly, samples were subjected to automatic precolumn derivatization using o-phthaldialdehyde with 2-mercaptoethanol (OPA Reagent, Agilent Technologies, Palo Alto, CA). Two eluents were used as mobile phases: eluent A (1.224 g of sodium acetate trihydrate, 500 mL of water, 0.09 mL of triethylamine, and 1.5 mL of tetrahydrofuran) and eluent B (1.088 g of sodium acetate trihydrate, 100 mL of water, 200 mL of acetonitrile, and 200 mL of methanol). A 65 min gradient program commenced with an initial concentration of 10% eluent B at a flow rate of 0.450 mL/min and terminated with 100% eluent B at a flow rate of 0.700 mL/min. The fluorescence wavelengths were 340 nm for excitation and 450 nm for emission. Supernatant samples were added of Norvaline (15 mg/L) as internal standard, diluted 1:1 with methanol and filtered through a 0.2 µm pore-size filter (Millipore) before injection. Injection volume was 5 µL.

**HPLC-DAD/ESI-MS.** A Hewlett-Packard series 1100 MSD (Palo Alto, CA) quadrupole mass spectrometer system equipped with an electrospray interphase (ESI) was used. Samples were introduced by direct injection. The ESI parameters were: drying gas N₂, 10 L/min at 330 ºC; nebulizer pressure, 40 psi; spray capillary voltage, 4000 V. The ESI was operated in negative mode, scanning from \( m/z \) 100 to \( m/z \) 800, using a variable fragmentator voltage gradient.

**RESULTS AND DISCUSSION**

Mycotoxins are well-known natural contaminants in foods. Among the more than 300 mycotoxins isolated and described until now, OTA is one of the most important because of its teratogenic, embryotoxic, genotoxic, neurotoxic, immunosuppressive, carcinogenic (IARC group 2B), and nephrotoxic effects (Joint FAO/WHO Expert Committee on Food Additives, JECFA) (29). Aside from the health and toxicological perspective, OTA
exposure also has an impact on livestock economics, where the associated decrease in productivity (milk, eggs, weight loss) and the increase of mortality rate are of concern (3). In fact, levels of OTA found in food products or beverages have been strictly fixed (ranging from 2-10 µg/kg) by the European Union (UE) in cereals, dried vine fruits, roasted coffee beans, ground coffee, soluble coffee, wine and grape juice (30), and the World Health Organization (WHO) has proposed a maximum limit of 5 µg/kg of OTA in cereals (31). In spite of it, and although biological decontamination of mycotoxins from foods by means of the use of microorganisms is one of the strategies used for the management of mycotoxins, little published information is available regarding biological methods for the removal of OTA.

To address this important point, and since it is known that soil bacteria are able to transform a wide range of aromatic compounds, we analyzed the presence of OTA in actinobacteria and *Pseudomonas* spp. grown in liquid BSM synthetic culture medium. Initially screenings of OTA degradation capacity were performed using *Pseudomonas putida* and several actinobacteria from the genera *Rhodococcus* and *Brevibacterium*. Bacterial strains were grown in liquid synthetic (BSM) culture medium in the presence of OTA (10 µg/L). As displayed in Table 1, only 8-28% decrease in OTA concentration was recorded in the cell-free supernatants for *Rhodococcus* and *Pseudomonas putida* strains, respectively, and no degradation products were observed on the HPLC chromatograms, suggesting that OTA is not degraded but adsorbed by the cells, as it has already been described for strains of lactic acid bacteria (27) and yeasts (32).

On the contrary, results in Table 1 also showed that OTA completely disappeared from the cell-free supernatants of *Brevibacterium casei* RM101 strain, indicative of the presence of a mechanism of OTA degradation. *Brevibacterium* spp. is a heterogeneous group of nine coryneform species that are capable of degrading insecticides (DTT, DDE, etc.), and produce self-processing
extracellular proteases. They are found in diverse habitats, including soil, poultry, fish, human skin, and food. *Brevibacterium* species differ from other bacteria for their ability to metabolize compounds of heterocyclic and polycyclic ring structure, a trait that is also common in fungi. Then, to analyze whether the above mentioned observed OTA degradation is a specific characteristic of *Brevibacterium* strain, species or genus, a media containing high concentration of OTA (40 µg/L, rarely found in food products or beverages) was used with strains belonging to different species from the *Brevibacterium* genus. A complete disappearance of OTA was observed in all the culture supernatants tested (Table 2), moreover, no traces of OTA were presented in pellets or pellet’s washing solutions after methanol extraction. Therefore, these results indicate that OTA degradation is a characteristic of the *Brevibacterium* genus.

Next, to further study the mechanism of OTA degradation followed by *Brevibacterium* spp., *B. casei* RM101 and *B. linens* DSM 20425T strains were grown at a 1000 times greater OTA concentration (40 mg/L), on different BSM medium compositions, including or not glycerol as carbon source. Results indicated that both *Brevibacterium* strains were able to completely degrade higher OTA concentrations (40 mg/L) also in a medium devoid of a traditional carbon source such as glycerol, where growth is slight, as determined by measuring the turbidity of the media (results not shown).

Analyses of the chromatograms from the supernatant showed that whilst the OTA peak was absent, a new peak with a different retention time and spectrum was present in the elution profile (Figure 2). In fact, the UV/Vis and fluorescence spectra of the produced compound found in the supernatants (Figure 3) were identical to the spectra corresponding to the OTα standard. Even more, HPLC-MS confirmed the identification of this compound, since the peak showed a molecular ion [M-H]– at m/z 255.1 in MS (PM OTα = 256).
As quantified by HPLC, similar amounts of OTα and PHE were presented in the supernatants of the two strains, B. casei RM101 and B. linens DSM 20425T, irrespectively of the presence or not of glycerol, and these quantities corresponded to the theoretical concentration calculated from the complete hydrolysis of the OTA added to the medium.

Furthermore, evaluation of the degradation of OTA over time showed that degradation already started 48 hours after inoculation of the strain (Figure 4A). Decrease of OTA concentration, production of OTα and growth of Brevibacterium casei RM101 in BSM media containing OTA, in the presence or absence of glycerol, was recorded (Figure 4).

These results strongly suggest that Brevibacterium spp. strains possessed an enzyme, possibly a carboxypeptidase, which hydrolyzed the amidic bond in the OTA molecule.

It has been reported that B. linens has highly active and multiple proteolytic enzymes that are mainly extracellular and intracellular proteases or peptidases (33, 34, 35). Electrophoretic studies have shown that sonicated extracts of a B. linens strain have at least six different peptide hydrolases of varying dipeptide specificities (36). In fact, zymograms obtained using 14 dipeptides of L-amino acids have shown that two peptides having a L-phenylalanine at the C-terminus (Ala-Phe, and Gly-Phe) were hydrolyzed by five extracts, whereas Ala-Trp and Ala-His dipeptides, containing the same N-terminus, were hydrolyzed just by one of the cell extracts assayed (36). Therefore, a possible carboxypeptidase activity acting on a peptide with L-phenylalanine as C-terminus can be found in B. linens extracts. Moreover, two aminopeptidases purified from a culture filtrate of a B. linens strain (37) that hydrolyzed a variety of substrates, have been shown to have specificity for N-terminal leucine. Additionally, one of the purified peptidases was also able to hydrolyze the tripeptide Phe-Phe-Phe with a relative activity of 49.1% compared to its activity on Leu-Leu; however, when a Phe residue in the D-configuration was added at the C-terminus, the activity decreases to 21.8%. One possible interpretation of this result is that the activity of the peptidase is higher when a L-Phe residue is located at the
C-terminus than when the residue is a D-Phe. Even though these data need to be further confirmed, it is worthy to note that the hydrolytic reaction against OTA exerted by bacteria of this genus could be probably due to a carboxipeptidase activity, since OTA is an isocumarin derivative linked, through the carboxyl group, to L-β-phenylalanine.

In the present study, our analysis showed a compound, OTα, identified by UV/Vis and fluorescence spectra and by HPLC-MS, as a product of the degradation of OTA by Brevibacterium strains. On the other hand, further HPLC analysis of supernatants in the conditions for the determination of amino acids showed the appearance of PHE in the theoretical concentration expected. These results are in agreement with previous data (12) indicating that the amidic bond present in OTA could be enzymatically hydrolyzed by CPA, rendering PHE and OTα, which is 500 times less toxic than OTA. The production of only OTα and PHE is interesting from the point of view of the possible use of Brevibacterium strains for detoxification of OTA in foodstuffs, as no other toxic degradation products were found.

In spite of many years of research and the introduction of good agricultural practices in food production and good manufacturing practices in the storage and distribution chain, mycotoxins continue to be a problem, and their impact in both human and animal health and welfare is wide-ranging. The usual methods to reduce the levels of OTA included physical-chemical washes, treatment with absorbent materials, solvent extraction, etc; however, these methods are expensive and can remove nutrients or important compounds from an organoleptic point of view. In this regard, to our knowledge, currently no biological treatment is being used to reduce the content of OTA in foods, beverages and feed, since the various microorganisms able to degrade it (see introduction) are not food-related microorganisms. In contrast, the genus Brevibacterium is widely used in food technology, as B. linens, B. casei and B. iodinum have been isolated from milk and cheese.
curd and contribute to the aroma, surface coloration and the ripening of several types of cheese (38, 39).

In this study we have demonstrated that Brevibacterium spp strains are able to totally degrade OTA, even at a concentration as high as 40 mg/L, a concentration 1000 times greater than the OTA concentration usually found in foodstuffs.

Since Brevibacterium spp. biomass can be produced, concentrated, freeze-dried, and possibly stabilized without losing its hydrolytic ability towards aromatic compounds (B. linens is commercially available as a starter for cheese ripening), the biological detoxification mechanism of OTA by Brevibacterium described here is therefore highly attractive and its use, for example as a feed additive, for mycotoxin degradation seems practicable. Even more, future elucidation of the genetic basis of the detoxification reaction and cloning of the corresponding gene(s) may contribute to the development of new enzymatic detoxification systems or to engineer this detoxification pathway in other organisms.

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Table 1. Reduction in concentration of OTA in BSM by Actinobacteria and Pseudomonas spp.

<table>
<thead>
<tr>
<th>Strain</th>
<th>OTA [µg/L]</th>
<th>OTA reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSM + OTA (Control)</td>
<td>11.01 (0.07)</td>
<td>0</td>
</tr>
<tr>
<td>*Rhodococcus erythropolis CECT 3008</td>
<td>7.88 (0.70)</td>
<td>28.47</td>
</tr>
<tr>
<td>*Rhodococcus erythropolis IGTS8</td>
<td>8.81 (0.98)</td>
<td>19.98</td>
</tr>
<tr>
<td>*Brevibacterium casei RM101</td>
<td>n.d.</td>
<td>100</td>
</tr>
<tr>
<td>*Pseudomonas putida DSM 291T</td>
<td>10.07 (0.09)</td>
<td>8.54</td>
</tr>
<tr>
<td>*Pseudomonas putida KT2442</td>
<td>8.18 (0.65)</td>
<td>25.70</td>
</tr>
</tbody>
</table>

n.d., not detected

*Results are expressed as mean values of two experimental replications for each strain; standard deviations are shown in parentheses.
Table 2. Reduction in concentration of OTA in BSM by *Brevibacterium* spp.

<table>
<thead>
<tr>
<th>Strain</th>
<th>OTA (µg/L)</th>
<th>OTA reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSM + OTA (Control)</td>
<td>39.81</td>
<td>0</td>
</tr>
<tr>
<td><em>Brevibacterium casei</em> DSM 20657&lt;sup&gt;T&lt;/sup&gt;</td>
<td>n.d.</td>
<td>100</td>
</tr>
<tr>
<td><em>Brevibacterium casei</em> DSM 9657</td>
<td>n.d.</td>
<td>100</td>
</tr>
<tr>
<td><em>Brevibacterium casei</em> DSM 20658</td>
<td>n.d.</td>
<td>100</td>
</tr>
<tr>
<td><em>Brevibacterium casei</em> RM101</td>
<td>n.d.</td>
<td>100</td>
</tr>
<tr>
<td><em>Brevibacterium linens</em> DSM 20425&lt;sup&gt;T&lt;/sup&gt;</td>
<td>n.d.</td>
<td>100</td>
</tr>
<tr>
<td><em>Brevibacterium iodinum</em> SM20626&lt;sup&gt;T&lt;/sup&gt;</td>
<td>n.d.</td>
<td>100</td>
</tr>
<tr>
<td><em>Brevibacterium epidermidis</em> DSM 20660&lt;sup&gt;T&lt;/sup&gt;</td>
<td>n.d.</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined from supernatants of three independent cultures

n.d., not detected
Figure 1. Conversion of OTA in L-β-phenylalanine and OTα
Figure 2: Chromatogram obtained from the supernatant of *B. casei* RM101 grown in BSM devoid of a carbon source but containing OTA (40 mg/L).

A) Supernatant at time 0, B) Supernatant after 10 days of growth showing the disappearance of the OTA peak and the appearance of the OTα peak.
Figure 3: OTα (A) UV/Vis and (B) fluorescence spectra obtained from the supernatant of B. casei RM101 grown in BSM devoid of a carbon source but containing OTA (40 mg/L).
Figure 4. (A) Decrease of OTA concentration and production of OTα and (B) growth of *B. casei* RM101 in BSM containing OTA (25 mg/L) with or without glycerol (0.2%).