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Degradation of Ochratoxin A by *Acinetobacter calcoaceticus*

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ABSTRACT

Microorganisms were screened for their ability to degrade ochratoxin A (OTA). Among test microorganisms, *Acinetobacter calcoaceticus* was found to degrade OTA. The degradation of OTA by *A. calcoaceticus* was studied in an ethanol-minimal salts medium with an initial OTA concentration of 10 μg/ml at 25 and 30°C. Under these conditions, *A. calcoaceticus* was able to degrade OTA with an initial concentration of OTA of 10 μg/ml. The average amounts of OTA removed by *A. calcoaceticus* in medium with an initial OTA concentration of 10 μg/ml were 0.1005 and 0.0636 μg/ml/h at 25 and 30°C, respectively. Ochratoxin A was degraded significantly by *A. calcoaceticus* during and after the log phase of cell growth at both incubation temperatures. It is postulated that degradation of OTA by *A. calcoaceticus* yielded a less toxic ochratoxin α.

Key Words: Degradation, Ochratoxin A, Ochratoxin α *Acinetobacter calcoaceticus*, mycotoxins, biodegradation

Generally, molds are not considered as agents of foodborne diseases but spoilage microorganisms. Following the outbreak of "turkey X" disease caused by aflatoxins in 1960, numerous studies have been conducted on toxins produced by molds, and the results revealed the carcinogenic, mutagenic and teratogenic effects of mycotoxins in animals and humans. Among mycotoxins, ochratoxins have attracted considerable attention, due to their toxic effects on biological systems.

Ochratoxins comprise a group of chemically related secondary metabolites, a 3,4-dihydromethyl isocoumarin moiety linked through the 7-carboxyl group to L-β-phenylalanine by an amide bond, and are produced by several species of *Aspergillus* and *Penicillium* (*17*). Among ochratoxins, OTA attracts the most extensive study, due to its occurrence and high toxicity in animals, while other members of ochratoxins, ochratoxin B and C, cause little or no acute toxicity. Ochratoxin A is found widely in foods of plant and animal origins, and it is toxic to cattle, swine, goats, chicken, turkey, duckling, rats, mice, dogs and fish. Ochratoxin A is of public health significance since it is associated with Balkan nephropathy, a kidney disease in humans (*11*). Cases of acute ochratoxicoses are characterized by nephropathy, enteritis and immunosuppressive symptoms (*10,14,19*). Under natural conditions, only OTA and, rarely, ochratoxin B have been encountered. Therefore, only OTA is considered a natural contaminant of foods and feeds (*2*). Ochratoxin A contamination in foods and feeds is considered a serious toxicological problem since OTA is capable of causing renal and hepatic carcinogenicity in mice (*6,7*).

Studies on microbiological degradation of OTA are not available. Although OTA has been reported to be degraded to non-toxic metabolites by rumen bacteria (*5,9*), the responsible bacteria have not been isolated and identified. The objectives of this study were to find microorganisms that are capable of degrading OTA and study the mechanism of microbial degradation of OTA. Results obtained from this study should provide information concerning the mechanism of microbial degradation of OTA and the possibility of using the microorganisms for degradation of OTA in food and feeds.

MATERIALS AND METHODS

Microbial screening for ochratoxin A degradation

Bacteria, yeasts and molds (Table 1), obtained from the Department of Food Science and Technology, The University of Tennessee, Knoxville, and several research institutes, were screened for ability to degrading OTA by using plate assay (*3*) and test tube assay methods. The test tube assay was a modification of the flask assay of Ciegler et al. (*3*).

Plate assay

In the plate assay, a modified Czapek-Dox medium (3.0% sucrose; 0.3% $NaNO_3$; 0.1% K_2HPO_4; 0.05% $MgSO_4$; 0.05% KCl; 0.001% $FeSO_4$; 0.005% yeast extract; 2.0% agar) (*3*), a non-fluorescent medium under ultraviolet (UV) light, was used as the growth medium for all test microorganisms. One milliliter of OTA (40 μg/ml in methanol) (Sigma Chemical Co., St. Louis, MO) was dispensed into petri dishes, and 18 ml of medium (55°C) was added to each petri dish and mixed evenly with OTA solution. Lids of petri plates were opened partially to dissipate methanol vapor. After the medium was solidified, plates were point inoculated with one loopful of test microorganism. Inoculated plates were incubated at 30°C and were examined under UV light at one day intervals for up to one week. Loss of fluorescence indicated possible detoxification.

Test tube assay

In this assay, media used for growing fungi and bacteria were malt yeast extract broth (0.3% yeast extract; 0.3% malt extract; 0.5% peptone; 1.0% dextrose) (*3*) and modified tryptose-glucose-

TABLE 1. List of microorganisms screened for ochratoxin A degradation.

	Plate assay	Test tube assay
Bacteria		
Acetobacter aceti	-[a]	-
Achromobacter xerosis NRRL B-1596	-	-
Acinetobacter calcoaceticus NRRL B-551	-	+[b]
Aeromonas hydrophila	-	-
Alcaligenes faecalis NRRL B-170	-	-
Arthrobacter oxydans	-	-
Azospirillum brasilense	NG[c]	-
Azospirillum lipoferum	-	-
Bacillus sp.	-	-
Bacillus cereus	-	-
Bacillus thuringiensis israelensis	-	-
Brevibacterium butanicum	-	-
Chromobacterium violaceum NRRL B-4369	-	-
Chromobacterium viscosum	-	-
Corynebacterium dioxydan	-	-
Escherichia coli NRRL B-3704	-	-
Flavobacterium auranticatum NRRL B-184	-	-
Flavobacterium devoran NRRL B-54	-	-
Gluconobacter oxydans	-	-
Klebsiella pneumoniae	-	-
Lactobacillus lactis	-	-
Lactobacillus plantarum C-11	-	-
Leuconostoc mesenteroides ATCC 8293	-	-
Micrococcus luteus	-	-
Microbacterium lacticum	-	-
Pediococcus pentosaceus FBB-61-6	-	-
Propionibacterium sp.	-	-
Proteus vulgaris	-	-
Pseudomonas aeruginosa	-	-
Pseudomonas fluorescens	-	-
Salmonella infantis	-	-
Selenomonas ruminantium	NG	-
Spirillum volutans	-	-
Staphylococcus aureus	-	-
Streptococcus bovis	NG	-
Streptococcus faecalis NRRL B-537	-	-
Xanthomonas campestris NRRL B-1459	-	-
Yeasts		
Candida albican NRRL Y-477	-	-
Hansenula sp.	-	-
Kluyverdmyces fragii	-	-
Pichia membranaefaciens	-	-
Rhodotorula sp.	-	-
Saccharomyces cerevisiae ATCC 9080	-	-
Saccharomyces ellipsoides	-	-
Saccharomyces rouxii	-	-
Torulopsis magnoliae	-	-
Zygopich chevaliereii	-	-
Molds		
Aspergillus niger	-	-
Aspergillus versicolor	-	-
Cladosporium	-	-
Fusarium semitectum	-	-
Geotrichum sp.	-	-
Mortierella sp.	-	-
Mucor sp.	-	-
Mucor attenvatus	-	-
Penicillium rubrium NRRL 6216	-	-
Penicillium urtical NRRL 994	-	-
Rhizopus nigricans	-	-
Spicaria violacea	-	-
Trichosporium sp.	-	-
Zygorhynchu ssp.	-	-

[a] Negative; [b] Positive; [c] No growth.

yeast extract broth (0.25% tryptone; 0.25% yeast extract; 1.0% glucose; 0.1% KH_2PO_4) (*3*), respectively. One milliliter of OTA was added to 9 ml of broth (20 µg/ml in 0.1 M sodium bicarbonate), and one loop of test microorganism was inoculated. Tubes were incubated at 30°C for 72 h. After incubation, cell-free filtrates (100 µl) were spotted on Redi-Plate silica gel G thin layer chromatography (TLC) plates (Fisher Scientific Co., Pittsburgh, PA). The TLC plates were developed in a solvent system containing benzene and acetic acid (9:1). After plates were developed and dried, they were observed under UV light. The degradation of OTA was indicated by absence of fluorescent spot or a weaker fluorescent spot than that of control on TLC plates. The degradation product of OTA also was indicated by fluorescent spots, which were identified as ochratoxin α. Ochratoxin α standard was obtained by hydrolyzing OTA with carboxypeptidase A (Sigma) (*13*).

Hydrolysis of ochratoxin A by A. calcoaceticus *in ethanol-minimal salts medium at 30°C*

Acinetobacter calcoaceticus NRRL B-551, obtained from Northern Regional Agricultural Research Service of United States Drug Administration (USDA, Peoria, IL), was found to degrade OTA. The degradation of OTA by this bacterium in a broth medium was studied. *Acinetobacter calcoaceticus* cells were grown in ethanol-minimal salts medium (pH 7.50) (*16*). Flasks containing 18.8 ml of ethanol-minimal salts medium and 1 ml of 0.1 M sodium bicarbonate with OTA concentrations of 0 or 0.2 mg/ml were inoculated with 0.2 ml 24-h culture of *A. calcoaceticus* to provide approximately 10^6 cells/ml and OTA concentrations of 0 and 10 µg/ml. Incubation was performed in a shaking water bath (Fisher) at 30°C in the dark. Flasks were agitated by reciprocal shaking at a rate of 90 strokes/min.

Enumeration of A. calcoaceticus

Microbial cell numbers were determined at 24 h intervals up to 168 h, a sample was withdrawn from flasks for analysis of cell numbers and residues of OTA. At each sampling period, serial dilutions were prepared with sterile 0.1% peptone water and spread-plated on pre-poured standard methods agar (Becton Dickinson and Co., Cockeysville, MD). Plates were incubated at 30°C for 48 h.

Degradation of ochratoxin A

To measure degradation of OTA, 0.5 ml sample was withdrawn from each flask. The reaction between cells and OTA was terminated by adding 0.2 ml of 1 N HCl (*5*). The sample was centrifuged in a Fisher micro-centrifuge (Fisher) at 12,400 rpm (136,000 × *g*) for 5 min. Supernatant was decanted and extracted with 2 ml of chloroform. The extraction was repeated three times or until fluorescence was not detected in the water phase. Extracts were pooled and filtered through an Acrodisc filter assembly (0.2 µm pore size) (Gelman Sciences, Ann Arbor, MI) and anhydrous sodium sulfate. Filtrate was passed through a Sep-Pak silica cartridge (Waters Associates, Inc., Milford, MA). The cartridge was washed with chloroform and flushed with nitrogen. Ochratoxin

A was eluted with methanol until no fluorescence was detected in eluent. The eluent was evaporated under nitrogen and redissolved in methanol to the original sample volume (*15,18*). Quantitation of OTA was carried out using high performance liquid chromatography (HPLC) (Waters) on a μ-porasil column with benzene-acetic acid-methanol (90:10:5) as eluting solvent at a flow rate of 1 ml/min. A model 420 fluorescence detector with a 340 nm excitation filter and a 440 nm emission filter (Waters) was used. The injection volume was 10 μL. Concentration of OTA was calculated based on fluorescent intensity compared to a standard curve obtained with OTA standards.

Statistical analyses

Data were analyzed by one-way analysis of variance (ANOVA) and interactive outlier rejection (linear regression) using Statgraphics (Statistical Graphic Co., Rockville, MD). Analysis of Variance with Tukey range test (*12*) was used to compare cell counts and OTA concentrations with a confidence level of 95% ($\alpha = 0.05$). Interactive outlier rejection was used to calculate the rate of OTA hydrolyzed by cells of *A. calcoaceticus* over incubation time. The overall rate of OTA hydrolysis was calculated by the regression of OTA changes over incubation time.

RESULTS AND DISCUSSION

Microbial screening

None of the test microorganisms (Table 1) tested with the plate assay caused a color change or the disappearance of fluorescence of OTA. Galtier and Alvinerie (*4*) reported that the disappearance of fluorescence of OTA could be due to the opening of the lactone group of the isocoumarin ring when OTA was hydrolyzed by caecal contents from rats. Since none of the test microorganisms caused the disappearance of OTA fluorescence, none of the test microorganisms had the ability to break the isocoumarin ring of OTA.

In the test tube assay (Table 1), *A. calcoaceticus* gave two fluorescent spots with blue and green color under UV light on TLC plates. The green and blue fluorescent spots had R_f-values of 0.69 and 0.33, respectively. Ochratoxin A standard had a R_f-value of 0.69. The blue fluorescent spot on TLC plates in *A. calcoaceticus* treatment was identical in fluorescent color and R_f-values to ochratoxin α.

An extracellular esterase has been found both in the cell-free growth medium and on the cell surface of *A. calcoaceticus* (*16*). Kaufman (*8*) reported that some soil microorganisms were capable of degrading phenylcarbamate herbicides (general formula, C_6H_5-NH-CO-R). Among these microorganisms, one isolate from *Acinetobacter* spp. was able to hydrolyze two common phenylcarbamate herbicides, 2-chloroethyl-N-(3-chlorophenyl)-carbamate (CEPC) and isopropyl-N-(3-chlorophenyl)-carbamate (CIPC). The degradation pathways of these two herbicides by soil microorganisms have been proposed to be the hydrolysis of the molecule at the amide bond or the ester linkage. Therefore, it is reasonable to assume that the mode of action of OTA degradation by *A. calcoaceticus* was similar to that of Acinetobacter breakdown of CEPC and CIPC (*8*); by hydrolysis of the amide bond. Since ochratoxin α was formed upon the degradation of OTA, results of the test tube assay strongly indicated that *A. calcoaceticus* degraded OTA to ochratoxin α by cleavage of the amide bond. However, further study is needed to confirm this hypothesis.

The fate of A. calcoaceticus *and the degradation of OTA in ethanol-minimal salts medium at 30°C*

The growth curve of *A. calcoaceticus* in ethanol-minimal salts medium without the addition of OTA at 30°C is shown in Fig. 1. The growth of *A. calcoaceticus* and the degradation of OTA in ethanol-minimal salts medium with an initial OTA concentration of 10 μg/ml at 30°C is shown in Fig. 2. The initial inoculum of *A. calcoaceticus* was approximately Log 5.6 CFU/ml. Cell numbers of *A. calcoaceticus* in growth medium with an initial OTA concentration of 10 μg/ml reached the maximum of Log 8.8 CFU/ml at 48 h (Fig. 2). Comparing the growth curve of *A. calcoaceticus* in medium containing 10 μg OTA/ml (Fig. 2) with that containing no OTA (Fig. 1), *A. calcoaceticus* cells decreased more rapidly after reaching a maximum in medium containing 10 μg/ml OTA. In medium containing 10 μg OTA/ml, cells increased after OTA was degraded (Fig. 2) indicating recovery of cells as toxin was removed from solution. It appeared that an OTA concentration of 10 μg/ml had a pronounced toxic effect on the cells of *A. calcoaceticus*, particularly at the end of the log phase. A growth inhibitory effect of OTA on micro-

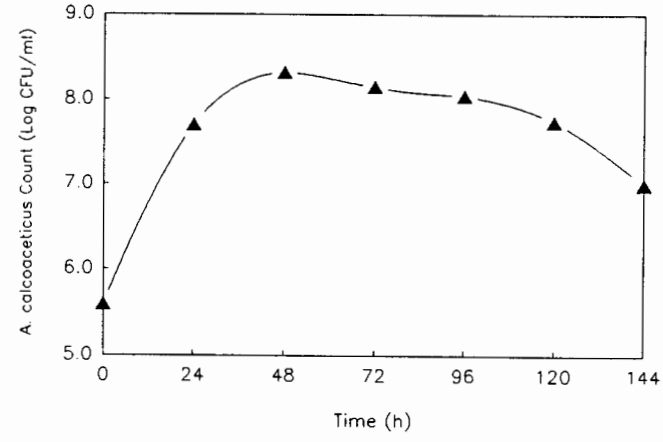

Figure 1. *Growth curve of* A. calcoaceticus *in ethanol-minimal salts medium at 30°C.*

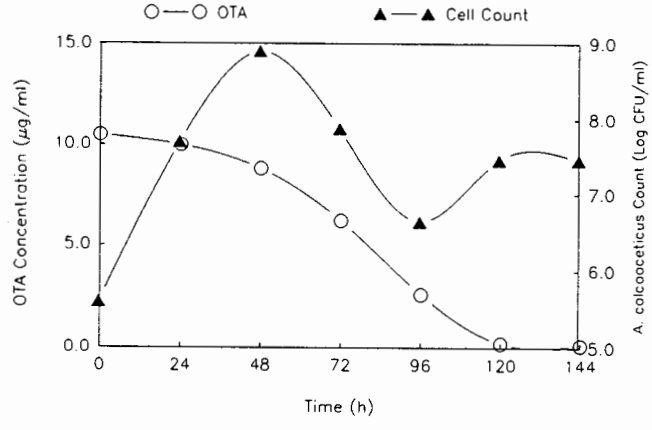

Figure 2. *Changes of ochratoxin A concentration and viable* A. calcoaceticus *in ethanol-minimal salts medium with ochratoxin A. concentration of 10 μg/ml at 30°C.*

organisms has also been reported by others (*1*).

With an initial OTA concentration of 10 μg/ml, OTA decreased significantly ($p < 0.05$) in the medium after 24 h. No OTA was detected in medium containing 10 μg OTA/ml after 120 h at 30°C (Fig. 2). The average decrease of OTA was 0.1005 μg/ml/h.

The fate of A. calcoaceticus *and the degradation of OTA in ethanol-minimal salts medium at 25°C*

The growth curve of *A. calcoaceticus* in ethanol-minimal salts medium without the addition of OTA at 25°C is shown in Fig. 3. The fate of *A. calcoaceticus* and the degradation of OTA in ethanol-minimal salts medium with an initial OTA concentration of 10 μg/ml at 25°C is shown in Fig. 4. The initial inoculum of *A. calcoaceticus* was approximately Log 6.0 CFU/ml. Cell numbers of *A. calcoaceticus* in growth medium with initial OTA concentrations of 10 μg/ml reached the maximum of Log 9.0 CFU/ml at 48 h following a 24-h lag phase (Fig. 4). The exponential growth patterns of *A. caleoaceticus* in ethanol-minimal salts medium with or without OTA at 25°C were similar to those at 30°C; however, a 24-h lag phase occurred at 25°C and no lag phase was observed at 30°C. In medium containing 10 μg OTA/ml, cell counts decreased slightly after inoculation but increased to the maximum after 48-h incubation. Ochratoxin A was seen to decrease significantly ($p < 0.05$) between 24 and 48 h, as cell count increased. In medium containing 10 μg OTA/ml, OTA significantly decreased ($p < 0.05$) at 48 h, and the decrease was linear ($r > 0.95$) thereafter. The residue of OTA in this medium was only 1 μg/ml after 144 h. The average decrease of OTA after *A. calcoaceticus* reached highest numbers (48-h incubation) was 0.0636 μg/ml/h (Fig. 4). Ochratoxin A was degraded much more rapidly ($p < 0.05$) in medium containing 10 μg OTA/ml at 30°C than at 25°C. This was probably due to the extended lag phase at 25°C. Ochratoxin A was not detected at 30°C after 120 h; however, small amounts of OTA remained. In our preliminary studies, *A. calcoaceticus* did not grow in minimal-salts medium (no ethanol added) containing OTA. This indicated that *A. calcoaceticus* was unable to use OTA as a sole carbon source. Therefore, ethanol was added to minimal salts medium to provide a carbon source in all experiments.

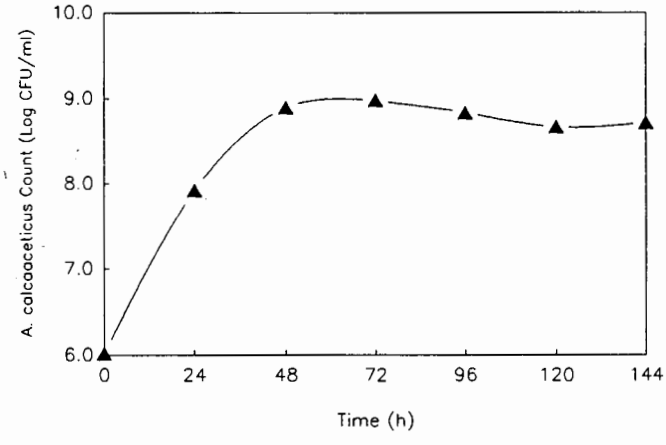

Figure 3. *Growth curve of* A. calcoaceticus *in ethanol-minimal salts medium at 25°C.*

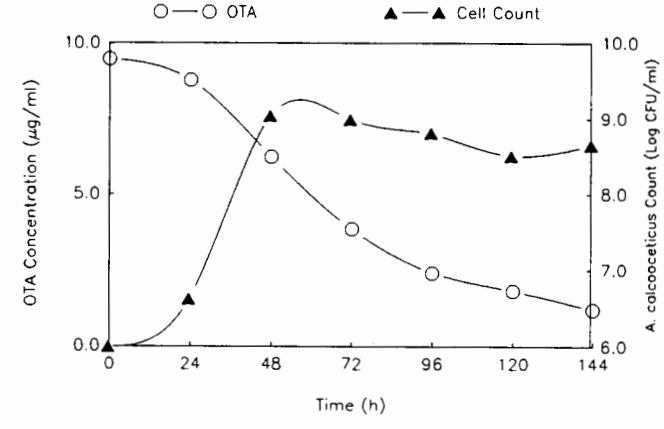

Figure 4. *Changes of ochratoxin A concentration and viable* A. calcoaceticus *in ethanol-minimal salts medium with ochratoxin A. concentration of 10 μg/ml at 25°C.*

SUMMARY AND IMPLICATIONS

Acinetobacter calcoaceticus were able to degrade OTA in ethanol-minimal salts medium with an initial OTA concentration of 10 μg/ml at both 25 and 30°C. The end product of OTA degradation by *A. calcoaceticus* was as ochratoxin α confirmed by TLC studies, although further confirmation is needed. Ochratoxin α has been shown to be much less toxic on chicken embryos than OTA (*19*). Therefore, the degradation of OTA by *A. calcoaceticus* may be considered a form of microbiological detoxication of OTA.

This study was performed under controlled conditions and an *in vitro* test system was used. The results of this study look promising; however, they do not imply the degradation of OTA in an *in situ* system using food or feed. The findings of this study provide incentive for further study of microbiological degradation of OTA. Studies are needed to characterize the chemical constituents involved in OTA degradation and to investigate the potential for degradation of OTA in foods and feeds. Studies are also needed to evaluate the toxicity of higher levels of OTA to *A. calcoaceticus*.

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