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ORIGINAL PAPER

An aminotransferase from bacterium ATCC 55552 deaminates hydrolyzed fumonisin B_1

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Abstract Previous research identified several microorganisms and pathways capable of degrading the mycotoxin fumonisin B_1 (FB_1). Degradation of FB_1 by microorganisms seems to comprise two essential steps: hydrolysis to hydrolyzed fumonisin B_1 (HFB_1) and deamination of the hydrolysis product. One of the previously studied microorganisms was the Gram negative bacterium ATCC 55552. The gene corresponding to the first step of FB_1 degradation in this bacterium was identified, but the genetic basis for deamination of the hydrolyzed intermediate remained unexplained (Duvick et al. 2000, PCT patent application WO200004158). Here we report the sequence and HFB_1-deaminating activity of a novel aminotransferase encoded by the bacterium ATCC 55552. The corresponding gene was identified, sequenced, and over-expressed in *Escherichia coli*. Cell lysates of the recombinant *E. coli* strain showed distinct HFB_1-deaminating activity in the presence of pyridoxal phosphate and pyruvate, as was demonstrated by liquid chromatography–mass spectrometry. Thus, we suggest the novel enzyme to be part of the fumonisin catabolic pathway of the bacterium ATCC 55552.

Keywords Aminotransferase · Bacterium ATCC 55552 · Deamination · HFB_1 · Hydrolyzed fumonisin B_1

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Introduction

Fumonisin B_1 (FB_1) is a mycotoxin produced by different phytopathogenic filamentous fungi that frequently contaminate corn and corn based products. It is the most abundant member of the fumonisins. Exposure to FB_1 by ingestion elicits equine leukoencephalomalacia (ELEM) in horses (Kellerman et al. 1990) and porcine pulmonary edema (PPE) in pigs (Harrison et al. 1990). FB_1 is suspected to promote esophageal cancer in humans (Marasas et al. 1988; Voss et al. 2002), and the degradation intermediate, hydrolyzed FB_1 (HFB_1), was shown to cause neural tube defects in cultured rat embryos (Flynn et al. 1997). The toxic effects caused by fumonisins are attributed to competitive inhibition of the enzyme ceramide synthase (Wang et al. 1991). The main chain bearing a C2-amino group and the tricarballylic acid (TCA) side chains of FB_1 mimic sphingoid bases and fatty acyl-CoAs, respectively, which are the substrates of ceramide synthase, and thus, FB_1 binds to the enzyme's active site (Merrill et al. 2001).

HFB_1, the hydrolysis product of FB_1, is a ten times weaker inhibitor of ceramide synthase than FB_1 (Merrill et al. 1993), but functions also as a substrate of this enzyme. Humpf et al. (1998) showed that HFB_1, by fatty acid acylation of the C2-amino group, gets converted into a ceramide analog, which is an even more competent inhibitor of ceramide synthase than FB_1. Acylated HFB_1 was shown in vitro to be about 10 times more toxic to mammalian HT-29 cells than FB_1. Acylation of HFB_1 also occurs in vivo (Seiferlein et al. 2007). Therefore, elimination of the TCA side chains is not sufficient for FB_1 detoxification, but also elimination of the C2-amino group is crucial.

In previous studies several microorganisms capable of degrading fumonisin B_1 have been identified (Benedetti et al. 2006; Blackwell et al. 1999; Duvick et al. 1998; Täubel 2005). Biodegradation of fumonisin B_1 in the black yeast *Exophiala spinifera* was shown to comprise two main steps: hydrolysis of FB_1 to HFB_1 by a carboxylesterase (Duvick et al. 1998) and deamination of HFB_1 by an amino oxidase (Blackwell et al. 1999).

Duvick et al. (2000) identified an FB_1 degrading gene cluster in the bacterium ATCC 55552. Whereas they elucidated the genetic basis of the first step of fumonisin B_1 degradation in this bacterium, hydrolysis of FB_1 to HFB_1 by a carboxylesterase, the gene responsible for deamination of HFB_1 was not yet examined adequately. They suggested two genes of the cluster, fccK and fccP possibly to encode for the FB_1 or HFB_1 deaminating enzyme of bacterium ATCC 55552. In preliminary studies we heterologously expressed these two genes in *Escherichia coli*, but did not find any FB_1 or HFB_1 deaminating activity of the recombinant enzymes (data not shown).

In a recent study, we reported a gene cluster (GenBank accession no. FJ426269) connected with FB_1 degradation in the bacterium *Sphingopyxis* sp. MTA144 (Heinl et al. 2010). Of the 11 proposed genes, one encoded an aminotransferase. Heterologous expression of this gene and determination of the activity of the recombinant protein confirmed that detoxification of HFB_1 by this strain is mediated by an aminotransferase, rather than an amino oxidase.

Here we report the sequence and HFB_1-deaminating activity of a novel aminotransferase encoded by the fumonisin degrading bacterium ATCC 55552, thereby contributing to a complete understanding of the FB_1 catabolic pathway of this microorganism, and showing that its catabolic pathway, contrary to *E. spinifera* and similar to *Sphingopyxis* sp. MTA144, is also based on an aminotransferase.

Materials and methods

Unless otherwise stated, all molecular methods and cloning techniques were performed according to Sambrook and Russell (2001).

Organisms, media and culture conditions

E. coli DH10B and *E. coli* BL21(DE3) (both from Invitrogen, Carlsbad, USA) were grown aerobically in Luria–Bertani (LB)-medium at 37°C. For selection of transformants, the medium was supplemented with 100 μg/ml ampicillin. The bacterium ATCC 55552 was obtained from the American Type Culture Collection and was grown aerobically in LB-medium at room temperature.

Enzymes, kits, and reagents

All enzymes, aside from the polymerase, were supplied by NEB (Ipswich, USA). Genomic DNA from bacterium ATCC 55552 was isolated using the DNeasy-kit from QIAGEN (Hilden, Germany). Plasmid DNA was extracted using the Wizard Plus SV miniprep kit (Promega, Madison, USA). PCRs were performed with the KOD DNA-polymerase (Novagen, Madison, USA) according to the manufacturer's recommendations. DNA for cloning was purified using the GFX-kit (GE Healthcare, Chalfont St. Giles, UK). All reagents were supplied by Sigma-Aldrich (Munich, Germany) unless specified otherwise. HFB_1 standard was supplied by Biopure Referenzsubstanzen GmbH, Tulln, Austria.

Nucleotide sequence elucidation

Degenerate primers based on the amino acid sequence of the HFB_1 deaminating aminotransferase from *Sphingopyxis* sp. MTA144, with a nondegenerate 5′-tail, representing the M13 or M13R primer sequence (Table 1), were designed according to Regier and Shi (2005). Degenerate PCR using the primers stated in Table 1 and genomic DNA from

Table 1 Primers used for degenerate PCR

Primer designation	Sequence (5′ → 3′)	Degree of degeneracy
M13-IPGGM (back)	TGT AAA ACG ACG GCC AGT TNA THC CNG GNG GNA TG	768
M13R-NGYPISA (for)	CAG GAA ACA GCT ATG ACC GCN GAD ATN GGR TAN CCR TT	768
M13	TGT AAA ACG ACG GCC AGT	Non-degenerate
M13R	CAG GAA ACA GCT ATG ACC	Non-degenerate

bacterium ATCC 55552 as template yielded a 700 bp DNA fragment. Sequencing of the fragment was performed using the primers M13 and M13R. The sequence of the flanking genome regions of the elucidated fragment were obtained by inverse PCR according to Ochman et al. (1988) using different walking primers. Sequencing was performed by Ibl GmbH, Vienna, Austria.

Construction of *E. coli* clones

The aminotransferase gene from bacterium ATCC 55552 was amplified by PCR, using genomic DNA as template and the following primers (restriction sites underlined): AT_55552_*Nde*I_back (5′-CAT CTA TCA TAT GGA ATT GAG CCG CCA ACG-3′) and AT_55552_*Bam*HI_for (5′-CAC TAT GGA TCC TTA GGC CGC TCT CAG GGC-3′). The PCR products were cloned using *Nde*I and *Bam*HI into the pET-3a vector (Novagen) yielding the plasmid pET-3a-AT55552. For propagation, the plasmid was introduced into *E. coli* DH10B by electroporation. For recombinant protein production, plasmid pET-3a-AT55552 was transferred into *E. coli* BL21(DE3), the resulting strain was designated *E. coli* BL21(DE3)AT55552.

Assay on enzyme activity and liquid chromatography-mass spectrometry (LC–MS) analysis

An overnight culture of *E. coli* BL21(DE3)AT55552 was diluted to an OD_{600} of 0.03 in 5 ml LB medium containing ampicillin. Cells were grown aerobically in a 50 ml Erlenmeyer flask at 25°C. After the cells reached an OD_{600} of 0.3, heterologous gene expression was induced by adding isopropyl β-D-thiogalactoside to a final concentration of 1 mM. After 24 h cells were harvested by centrifugation for 5 min at 8,000 rcf. The cells were lysed by sonication in the original culture volume of 50 mM sodium phosphate buffer (pH 8.0) at 0°C. Expression of the recombinant gene was confirmed by SDS-PAGE (10% polyacrylamide gel, 200 V). Deamination reactions were performed as follows: 4723 µl sodium phosphate buffer (50 mM, pH 8.0), 600 µl 10× deamination buffer (50 mM sodium phosphate buffer, pH 8.0, 200 µM pyridoxal phosphate, 30 mM pyruvate) and 77 µl HFB_1 standard solution (392 µg/ml, resulting in a final nominal HFB_1 concentration of 12.4 µM), were mixed with aliquots of 600 µl of the crude cell lysate and incubated at 25°C. Samples of 100 µl were taken immediately after addition of the cell lysate ($t = 0$ h), after 15, 22 and 44 h and were heat inactivated for 5 min at 99°C. HFB_1 concentrations were measured by LC–MS by Quantas Analytics GmbH (Tulln, Austria) as described previously (Heinl et al. 2010).

Results and discussion

DNA sequence and prediction of the coding region

In an earlier study, we identified and sequenced an HFB_1 deaminating aminotransferase encoded within a gene cluster of the bacterium *Sphingopyxis* sp. MTA144. Based on the amino acid sequence of this aminotransferase, degenerate primers were designed. Degenerate PCR yielded a DNA fragment of 700 bp length. The deduced amino acid sequence of one reading frame showed similarity to glutamate-1-semialdehyde aminotransferases. The nucleotide sequence of adjacent genome regions was obtained by inverse PCR and primer walking. Overall, the sequence of a region of 1,288 bp was elucidated and was submitted to the NCBI database (GenBank accession no. GU338977). Analysis of the obtained nucleotide sequence using the pDRAW32 software

(http://www.acaclone.com) revealed one putative gene, encoding a polypeptide of 423 amino acids with a calculated molecular weight of 46.24 kDa and a p*I* of 5.48. A putative ribosomal binding site was found upstream of the translation start (see Fig. 1).

BLAST analysis

BLAST analysis (Altschul et al. 1990) of the deduced amino acid sequence (blastp) of the identified ORF displayed 75% similarity (64% identity) to the amino acid sequence of the HFB_1 deaminating aminotransferase from *Sphingopyxis* sp. MTA144, and 59% similarity (41% identity) to an FB_1 deaminating aminotransferase (SEQ ID NO: 56 from patent WO2004085624), obtained from an environmental sample (Zhao et al. 2004). Search of the deduced amino acid sequence of the aminotransferase gene against the NCBI non-redundant protein sequence database (nr) and the patented protein sequences database (pat) revealed further similar sequences: The glutamate-1-semialdehyde-2,1-aminomutase from *Hyphomonas neptunium* ATCC 15444 (GenBank accession no. ABI75653.1) shares 75% similarity (60% identity), and sequence 10877 from patent US 7,314,974 (GenBank accession no. ABZ36939.1) from an unknown organism shares 75% similarity (61% identity) with the amino acid sequence of the aminotransferase from ATCC 55552. Whether FB_1 or HFB_1 is also a substrate of these enzymes is presently unknown.

Activity assay: deamination of HFB_1

The aminotransferase gene isolated from ATCC 55552 was heterologously expressed in *E. coli* and crude cell lysate of the recombinant strain was subjected to HFB_1 deamination assays. Decrease of HFB_1 concentrations was determined by LC–MS analysis. The deamination assay showed that the recombinant aminotransferase was able to deaminate HFB_1 in the presence of pyruvate and pyridoxal phosphate. Nearly complete deamination of 8.9 μM HFB_1 by cell lysate derived from IPTG induced *E. coli* BL21(DE3)AT55552 cells was detected after 15 h (Fig. 2A). Figure 2B shows that no HFB_1 deamination occured with cell lysate from the

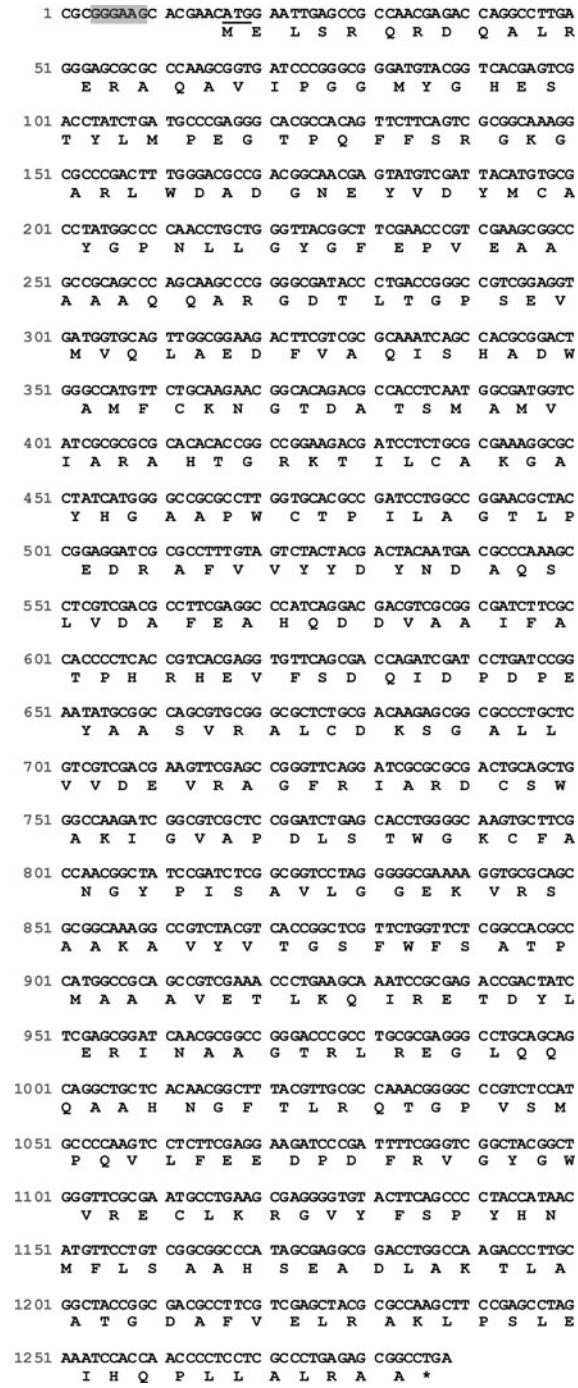

Fig. 1 Nucleotide sequence and deduced amino acid sequence of the aminotransferase from bacterium ATCC 55552. The putative ribosomal binding site is highlighted in *gray*, the start codon is *underlined*, the termination codon is indicated by *asterisk*

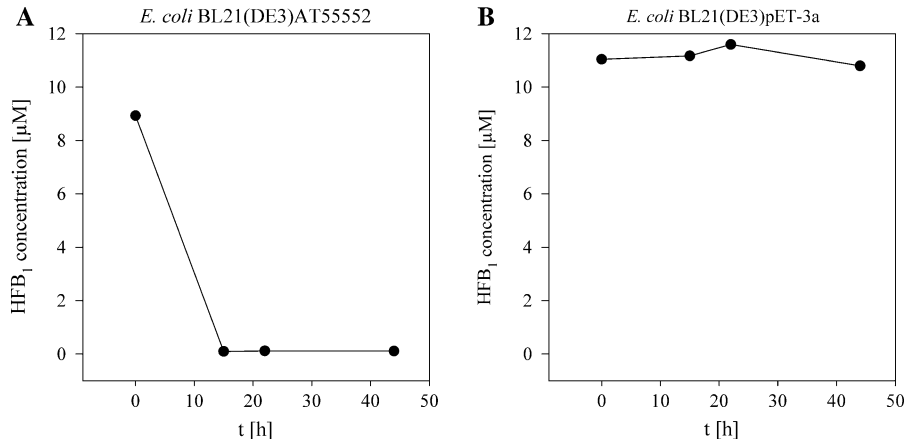

Fig. 2 LC–MS analysis of HFB_1 deamination by cell lysates of (**A**) *E. coli* BL21(DE3)AT55552, expressing the aminotransferase gene from bacterium ATCC 55552, and of (**B**) the negative control *E. coli* BL21(DE3)pET-3a, carrying only the empty pET-3a vector. Concentrations of HFB_1 are plotted versus incubation time

negative control *E. coli* BL21(DE3), carrying the empty pET-3a vector.

Whereas in the fumonisin degrading black yeast *E. spinifera* deamination of HFB_1 is catalyzed by an amino oxidase, both bacterium ATCC 55552 and *Sphingopyxis* sp. MTA144 use an aminotransferase for HFB_1 deamination. The bacterial enzymes have a higher potential for technological applications in fumonisin detoxification, since, contrary to amino oxidases, they are independent of molecular oxygen. Therefore it may be possible to use them for HFB_1 deamination under anaerobic conditions, such as predominate in silages or in the intestinal tract of animals.

Conclusions

Here we report the sequence and HFB_1-deaminating activity of a novel aminotransferase encoded by the fumonisin degrading bacterium ATCC 55552. The identified gene was expressed heterologously in *E. coli* and the recombinant enzyme was shown to deaminate HFB_1 in the presence of the α-keto acid pyruvate and the coenzyme pyridoxalphosphate. The subsiding transamination reaction is independent of molecular oxygen, which could be advantageous for particular technological applications. By identifying the as yet missing genetic basis of HFB_1 deamination in bacterium ATCC 55552, we contribute to the complete understanding of the FB_1 catabolism of this organism. Since HFB_1 aminotransferase genes were confirmed in bacterium ATCC 55552 and *Sphingopyxis* sp. MTA144, HFB_1 deamination by an aminotransferase, rather than an amino oxidase like in *E. spinifera* (Blackwell et al. 1999), may be the preferred fumonisin degradation pathway in bacteria.

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