Isoenzyme N-Acyl-L-Amino Acid Amidohydrolase NA Increases Ochratoxin A Degradation Efficacy of Stenotrophomonas sp. CW117 by Enhancing Amidohydrolase ADH3 Stability

Nan Chen,a Qingru Fei,a Han Luo,a Zemin Fang,b Yazhong Xiao,b Zhengjun Du,a @Yu Zhoua,b

aState Key Laboratory of Tea Plant Biology and Utilization, School of Tea and Food Science Technology, Anhui Agricultural University, Heifei, China
bSchool of Life Sciences, Anhui University, Hefei, China

Nan Chen and Qingru Fei contributed equally to this work. The order is based on workload and difficulty of the work.

ABSTRACT Ochratoxin A (OTA) is a potent mycotoxin mainly produced by toxigenic strains of Aspergillus spp. and seriously contaminates foods and feedstuffs. OTA detoxification strategies are significant to food safety. A superefficient enzyme ADH3 to OTA hydrolysis was isolated from the difunctional strain Stenotrophomonas sp. CW117 in our previous study. Here, we identified a gene N-acyl-L-amino acid amidohydrolase NA, which is an isoenzyme of ADH3. However, it is not as efficient a hydrolase as ADH3. The kinetic constant showed that the catalytic efficiency of ADH3 (Kcat/Km = 30,3938 s⁻¹ · mM⁻¹) against OTA was 29,113 times higher than that of NA (Kcat/Km = 10.4 s⁻¹ · mM⁻¹), indicating that ADH3 was the overwhelming superior detoxifying gene in CW117. Intriguingly, when gene na was knocked out from the CW117 genome, degradation activity of the Δna mutant was significantly reduced at the first 6 h, suggesting that the two enzymes might have an interactive effect on OTA transformation. Gene expressions and Western blotting assay showed that the Δna mutant and wild-type CW117 showed similar adh3 expression levels, but na deficiency decreased ADH3 protein level in CW117. Collectively, isoenzyme NA was identified as a factor that improved the stability of ADH3 in CW117 but not as a dominant hydrolase for OTA transformation.

IMPORTANCE Ochratoxin A (OTA) is a potent mycotoxin mainly produced by toxigenic strains of Aspergillus spp. and seriously contaminates foods and feedstuffs. Previous OTA detoxification studies mainly focused on characterizations of degradation strains and detoxifying enzymes. Here, we identified a gene N-acyl-L-amino acid amidohydrolase NA from strain CW117, which is an isoenzyme of the efficient detoxifying enzyme ADH3. Isoenzyme NA was identified as a factor that improved the stability of ADH3 in CW117 and, thus, enhanced the degradation activity of the strain. This is the first study on an isoenzyme improving the stability of another efficient detoxifying enzyme in vivo.

KEYWORDS food safety, mycotoxin pollution, codegradation, detoxification, isoenzyme, ochratoxin A
Detoxification studies have shown that OTA is mainly transferred by three possible pathways (i.e., lactone ring opening, producing OP-OTA; isocoumarin ring dechlorinating, producing OTB; or peptidic bond cleaving, producing OTα and L-β-phenylalanine) (8–11). Toxicological evaluation showed that OTα is a much less toxic metabolite compared to the parent chemical OTA, followed by OTB and OTA lactone-opened product (OP-OTA) (10–11).

Among the detoxification methods, biodetoxification by microbial strains and bio-enzymes has received much attention for serial merits (environmentally friendly, low cost, and high specificity). However, most of the biodetoxification studies focused on degradation strain screening and characterization (12). In addition, some commercial hydrolases, such as carboxypeptidase and peptidase, were characterized for OTA detoxification, but the efficiencies were relatively low for further industrial development (13–14). Recently, several OTA-detoxifying enzymes (or genes) have been identified, and carboxypeptidases are the most studied. Among the OTA-detoxifying enzymes, the amidohydrolase ADH3 from Stenotrophomonas sp. CW117 and ochratoxinase (i.e., OTase) from Aspergillus niger were the most efficient and investigated hydrolases, and OTase was the first crystal structure characterized detoxifying enzyme that was highly significant to understanding the detoxifying mechanism (11, 15). Moreover, the activities of amidohydrolase ADH3, OTase, and the N-acyl-α-amino acid amidohydrolase (i.e., AFOtase) from Alcaligenes faecalis, were much more efficient than bovine pancreatic carboxypeptidase (CPA) (the first OTA-detoxifying enzyme) and other characterized hydrolases (11, 16).

Despite OTA-detoxifying strains and enzymes having been widely investigated, only a few studies focused on OTA-degrading genes (or purified enzymes) screening and characterization. Meanwhile, several studies found that the identified detoxifying enzyme showed much lower activity than the host strain from which the enzyme (or gene) was isolated (9, 17–19). The studies on OTA degradation by Lysobacter sp. CW239 found that strain CW239 degrades OTA by joint action of multiple detoxifying enzymes, and the identified carboxypeptidase CP4 showed only limited contribution to OTA degradation in the strain; unfortunately, efficient detoxifying enzymes (or genes) remained unknown (9, 20). Other than Lysobacter sp. CW239, no further study tried to illustrate potential reasons for the activity disparity between the identified enzyme and host strain, especially of degradation mechanisms in these degradation strains.

RESULTS

OTA-detoxifying enzymes ADH3 and NA from CW117. As described in a previous publication, 53 hydrolase genes, which may potentially be used for OTA detoxification, were selected for degradation testing from the CW117 genome (GenBank accession no. CP062156.1) (11). Two genes, adh3 (protein, ADH3) and na (protein, NA), were screened and characterized for OTA degradation activity by heterologous protein expression in Escherichia coli BL21 and enzymatic degradation test in vitro. Open reading frames (ORFs) of two genes that encode enzymes NA and ADH3 were amplified from the CW117 genome, ligated to vector pGEX-4T-1, and transferred into E. coli BL21 for protein expression. Gene adh3 has been fully characterized in Luo et al. (11), the repeated ADH3 results were not shown (or only as a parallel comparison) in this study. As shown in Fig. 1, gene na (1,317 bp) was cloned from the CW117 genome and recombinant enzyme NA was expressed by E. coli BL21. Based on the sequence analysis and protein prediction of online programs (Expasy, ProtParam tool), NA is classified as N-acyl-α-amino acid amidohydrolase and composed of 438 amino acids, with a predicted molecular weight (MW) of 46.4 kDa and isoelectric point (pI) of 6.1. As shown in Fig. 1A, the purified recombinant N-acyl-α-amino acid amidohydrolase (rNA) by GSTrap FF columns showed an apparent MW of 72.4 kDa (including 26 kDa glutathione S-transferase [GST]-tag),
which was consistent with online predictions. Meanwhile, amidohydrolase ADH3 is composed of 427 amino acids, the theoretical molecular weight is 45.6 kDa, and the isoelectric point is 6.9. Enzymatic activity tests showed about 60% of OTA was degraded by 0.1 mg/L purified rNA within 12 h, while 25 μg/L OTA was completely degraded by rADH3 in only 120 s with much less protein (1.2 μg/L) (Fig. 1B).

Optimal temperature and pH tests showed that rNA showed greater pH adaptation (pH 2 to 10) than rADH3, but rADH3 showed significantly greater thermal adaptability to rNA (see Fig. S1A and B in the supplemental material). In addition to Li⁺, 0.05 mol/L metal ion Cu²⁺, Zn²⁺, Fe³⁺, Ca²⁺, or Mg²⁺ showed significant inhibition to rADH3 activity; however, metal ion Cu²⁺, Zn²⁺, Fe³⁺, or Ca²⁺ improved rNA activity to OTA degradation, and only Li⁺ and Mg²⁺ showed significant inhibition to rNA activity (Fig. S1C). Two detoxifying enzymes are sensitive to proteases and protein denaturants (1% SDS, proteinase K, or 1% SDS plus proteinase K), but the metal-chelator EDTA showed no inhibition to rADH3 (Fig. S1D). In enzymatic characterization, two detoxifying enzymes NA and ADH3 showed quite different physical and chemical properties from each other, which might enable strain CW117 to degrade OTA in different environmental conditions.

**Phylogenetic results on OTA-detoxifying enzymes and host organisms.** Phylogenetic analysis of detoxifying enzymes showed that previously identified OTA-detoxifying enzymes were mainly distributed in two superfamilies of amidohydrolase and carboxypeptidase. Hereinto, detoxifying enzymes with significant activity (Kcat/Km > 10.0 s⁻¹ mM⁻¹) were mainly affiliated with the amidohydrolase superfamily, but detoxifying enzymes with relative low activity were from the carboxypeptidase superfamily (see Fig. S2 in the supplemental material). Detoxifying enzyme NA (gene locus tag H7691_01355) was closest to N-acyl-L-amino acid amidohydrolase AfOTase, detoxifying enzyme ADH3 (gene locus tag H7691_12935) was closest to amidohydrolase superfamily OTase, and the two detoxifying enzymes formed separated clades with their closest neighbors (Fig. S2). Phylogenetic analysis of the hosts of detoxifying enzymes found that microbial strains can contain 2 to 3 detoxifying enzymes, but animals contain only one detoxifying CPA and the efficient detoxifying enzymes (e.g., ADH3, OTase, and AfOTase) distributed in four microbial clusters (I to IV) (Fig. 2). Cluster I contains up to three genes, two of which are similar to efficient detoxifying enzymes (NA and ADH3), and cluster II contains two genes, one of which is similar to an efficient detoxifying enzyme (ADH3). Strains of clusters I and II should be the most efficient OTA-detoxifying organisms, which contain more than two genes with at least one gene similar to super-efficient enzyme ADH3. The other two clusters (i.e., III and IV) contain only one gene, which is similar to the less efficient enzyme AfOTase or OTase. Interestingly, the organisms from clusters I and II are members of Xanthomonadales, and those from cluster III are members of Burkholderiales, which are affiliated with phylum proteobacteria (Gram-negative bacteria). In addition to Aspergillus spp. of cluster IV, which contain a gene similar to OTase, other efficient OTA degradation organisms almost distribute in phylum proteobacteria. Although detoxifying enzymes from the carboxypeptidase

![FIG 1](image-url) Protein expression and OTA degradation activity. (A) SDS-PAGE analysis of purified rNA (M, marker; lane 1, the unpurified protein sample; lane 2, the purified rNA). (B) Degradation activity of rADH3 and rNA.
superfamily have been extensively identified and characterized from Gram-positive bacteria (e.g., *Acinetobacter* spp., *Alkalihalobacillus* spp., and *Bacillus* spp.) and some animals (e.g., *Bubalus bubalis*, *Ovis aries*, *Bos taurus*), their activities were substantially limited (Fig. 2).

Degradation dynamics and product identification. OTA degradation product from rNA was purified using an OchraTest column (Vicam, Milford, MA, USA) and identified by ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using the same procedures as Luo et al. (11). The OTA degradation product from rNA produced [M + H]+ at m/z 257 as a parent ion from the full scan MS spectrum, and the parent ion produced [M + H]+ at m/z 167 and 211 as daughter ions in MS/MS spectrum, which was consistent with the rADH3-degraded product OTα discussed previously (Fig. 3A to D). Dynamics of OTA degradation and OTα production showed that 0.1 μmol/L OTA was transformed to OTα by 0.1 mg/L rNA within 48 h (Fig. 3E to G), while, rADH3 completed the degradation process within 2 min by 1.2 mg/L protein (11). During OTA degradation, the molar equivalent of OTA residue plus OTα production remained constant (0.1 μmol/L), indicating that the degraded OTA was completely transformed to OTα (Fig. 3G). Kinetic constant showed that $K_m$ values of rNA and rADH3 were 0.0038 mM and 0.000039 mM, and the $K_{cat}/K_m$ values of the two enzymes were 10.4 and 30,3938 s⁻¹ · mM⁻¹, respectively. Detoxifying enzyme rADH3 exhibits an extremely high activity to OTA transformation, and the catalytic efficiency ($K_{cat}/K_m$) was 29,113 times higher than that of rNA. In addition, the catalytic efficiency of rADH3 was even 210 and 56.7 times higher than those of rOTase from *Aspergillus niger* and AfOTase from *Alcaligenes faecalis*, which were previously recognized as the most efficient detoxifying enzymes (11, 15, 16). Other than degradation efficiency, the enzymes rNA and rADH3 showed the same degradation mechanism of amide bond hydrolysis (Fig. 3E), and the two hydrolases were the isoenzymes from strain CW117.
Construction and screening results of mutants and complementary strains. By PCR amplification, the upstream of gene na (USna) (792 bp) and downstream of gene na (DSna) (913 bp) fragments that flank the na were amplified, and fragments of USna and DSna were ligated by overlap PCR with the US-DSna fragment of 1,705 bp (see Fig. S3A in the supplemental material). After that, the purified US-DSna was cloned to pK18mobSacB and validated (Fig. S3A). By verifying primers of val1-F/R, the PCR product from the Dna mutant was expected to be 1,967 bp, but PCR product from wild-type CW117 was expected to be 3,286 bp (Fig. S3A). As shown in Fig. S3B, the USadh3 (739 bp) and DSadh3 (718 bp) fragments flanking the gene adh3 were amplified, and the two fragments were ligated by overlap PCR. By verifying primer pair val3-F/R, PCR product from the Dadh3 mutant (or Dna-adh3 double mutant) was expected to be 2,292 bp, but PCR product from CW117 (or starting strain Dna), which contains adh3, was 3,654 bp (Fig. S3B).

Based on mutant construction, the gene (na or adh3) complementary strain was further constructed and tested for phenotype recovery (i.e., OTA degradation). As illustrated in Fig. S3C and E, the complete ORF of na or adh3 was cloned (1,317 or 1,284 bp), and the recombinant plasmid pSRK-Gm/na (or pSRK-Gm/adh3) was successfully constructed. The recombinant plasmid pSRK-Gm/na or pSRK-Gm/adh3 was transformed to the Dna-adh3 double mutant, and the (Dna-adh3)/na and (Dna-adh3)/adh3 complementary strains were obtained, respectively (Fig. S3D/F).

Growth characters and ROS levels in wild-type and mutants. As illustrated in Fig. 4A, growth characteristics of the Dna-adh3 and Dna mutants showed no significant difference to wild-type CW117, indicating that genes na and adh3 deficiency did not change the bacterium growth characteristics. Reactive oxygen species (ROS) are recognized as an important physiological indicator for apoptosis and antimicrobial infections, and ROS homeostasis is significant to the health of bacteria (21, 22). Whenever under low or high OTA content, the ROS level of wild-type CW117 showed no significant difference from the Dna-adh3 and Dna mutants (Fig. 4B and G); this result indicated that na and adh3 deficiency did not change ROS homeostasis of CW117. However, at high OTA content, the ROS levels of CW117 and mutants were equally reduced after 12 h of incubation, and a significant difference was observed for strain CW117 ($P < 0.05$), indicating that 500 $\mu$g/L OTA showed cytotoxicity to strain CW117.

FIG 3 The degradation characteristic of N-acetyl-L-amino acid amidohydrolase rNA in vitro by UPLC-MS/MS under the positive ionization mode. Mass spectra of OTA (A), MS/MS spectra of OTA (B), mass spectra of OTA degradation product (C), MS/MS spectra of OTA degradation product (D), catalyzing mechanism of rNA against OTA (E), HPLC chromatogram of degrade product and OTA residue (F), dynamics of OTA degradation and OTA production (G).
Degradation genes verify in vivo. Before degradation testing, the mutants and complementary strains were further validated by PCR sequencing. As shown in Fig. 5A, the mutants and complementary strains were correctly constructed. The \( \Delta na \) mutant showed similar OTA degradation dynamic to wild-type CW117, except at the time point of the 6th hour, from which the degradation ratio was significantly lower than that of CW117 (\( P < 0.001 \)). Compare to that of the \( \Delta na \) mutant, the degradation activity of the \( \Delta adh3 \) mutant was reduced dramatically, and the degradation ratio of the \( \Delta adh3 \) mutant was less than 10% at the 9th hour. However, the \( \Delta na-adh3 \) double mutant lost OTA degradation activity completely (Fig. 5B). Mutant validation results indicated that genes \( na \) and \( adh3 \) are the only OTA-detoxifying genes in strain CW117, and \( adh3 \) is much more efficient than \( na \). OTA degradation testing of \( na \) and \( adh3 \) in vivo was consistent with the activity of detoxifying enzymes rADH3 and rNA in vitro.

In complementary strain testing, we found that both the \( \Delta na-adh3/na \) and \( \Delta na-adh3/adh3 \) strains displayed OTA degradation ability. Thereinto, the \( \Delta na-adh3/na \) strain, which contains gene \( na \) recovered only partial activity compared to that of CW117. However, the \( \Delta na-adh3/adh3 \) strain, which contains one gene \( adh3 \) showed a significant higher degradation ratio (\( P < 0.001 \)) than wild-type CW117 at the 12th hour (Fig. 5D). Meanwhile, the expressed ADH3 protein at the 12th hour of the degradation process was examined by Western blotting using the polyclonal antibody prepared previously (see Fig. S4 in the supplemental material). In contrast to OTA degradation results, Western blotting showed that both CW117 and the \( \Delta na-adh3/adh3 \) strain produced detoxifying enzyme ADH3, and the \( \Delta na-adh3 \) mutant did not produce ADH3 (Fig. 5C); under 10 \( \mu \)g of total protein loading, the expressed ADH3 protein in the \( \Delta na-adh3/adh3 \) strain that was induced by 0.1 mM isopropyl-\( \beta \)-1-thiogalactopyranoside (IPTG) was significantly higher than that of wild-type CW117 (Fig. 5C). Hence, the high expression level of ADH3 should contribute to higher degradation activity for the complementary \( \Delta na-adh3/adh3 \) strain. These results demonstrated that both NA and ADH3 showed OTA degradation activity in vivo, and the ADH3 was the crucial detoxifying enzyme that showed much higher activity than NA in strain CW117.

Joint degradation actions of \( na \) and \( adh3 \). Degradation activity in vitro showed that rADH3 catalytic efficiency (\( K_{cat}/K_m \) value) was 29,113 times than that of rNA, and the activity difference of the two enzymes was validated in vivo. If we compared the degradation results
in vitro, the degradation contribution of enzyme NA in strain CW117 should be very limited and can be neglected. However, when compared to wild-type CW117, the degradation activity of the Δna mutant was reduced significantly \( (P < 0.001) \) in the initial 6 h \( (\text{in vivo}) \) during degradation (Fig. 5B). This result indicated that gene na deficiency significantly reduced degradation activity of CW117 in the first 6 h, but the growth character and ROS of the Δna mutant showed no difference from wild-type CW117 (Fig. 4). Corresponding to OTA degradation results, the gene expressions of na and adh3 in the Δna mutant and wild-type CW117 were examined at the 6th hour during the OTA degradation process. Real-time quantitative PCR (qPCR) showed that na not expressed in the Δna mutant compared to wild-type CW117; however, the adh3 expression level in the Δna mutant did not show a significant difference from that of the wild-type CW117 (Fig. 6A). In contrast to RNA levels, the ADH3 protein content in wild-type CW117 determined by Western blotting was significantly higher than that of Δna in the first 6 h. That is, na deficiency did not influence the adh3 RNA level but reduced the ADH3 protein level (Fig. 6B). The comparison result of gene expressions and ADH3 protein contents in the Δna mutant and wild-type CW117 indicated that isoenzyme NA did not influence adh3 expression but improved the stability of ADH3 in CW117 and thus increased OTA degradation activity of the strain \( \text{in vivo} \).

In addition, substrate induction characters were examined for the expression of the two genes. When strain CW117 was cultured at the mid-log phase (as shown in Fig. 4A) and induced by 50 and 100 μg/L OTAs for 40 min, the expressions of the two genes (na and adh3) were upregulated, and significant differences were observed for the 100-μg/L OTA group (Fig. 6C). Compared to RNA levels, the expressed ADH3 protein content of CW117 was gradually enhanced by increasing OTA (Fig. 6D). Substrate induction results by reverse-transcription quantitative PCR (RT-qPCR) and Western blotting indicated that the two genes are substrate inducible, especially under the higher content of 100 μg/L. Moreover, we should realize that na and adh3 are the constitutive expression genes in CW117, and the two genes were constitutively expressed without OTA induction (controls in Fig. 6C and D). That is to say, other than OTA degradation, the two genes might have other physiological functions, such as amino acids metabolism and
2-oxocarboxylic acid metabolism annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

**DISCUSSION**

In this study, two OTA degradation genes, *na* and *adh3*, were screened and compared from *Stenotrophomonas* sp. CW117, and the degradation activity was verified by *in vitro* and *in vivo* tests. Other than NA and ADH3, no other degradation agent exists in strain CW117 (Fig. 5B). Strain CW117 degrades OTA through amido bond cleavage by the isoenzymes of *N*-acyl-L-amino acid amidohydrolase NA and/or amidohydrolase ADH3, and both of the detoxifying enzymes showed the same degradation mechanism (Fig. 3E), and the pathways that produce product OTA have been recognized as the most effective manner on OTA detoxification, followed by pathways of OTB and OP-OTA as the detoxifying products (8, 10, 11). Degradation verification *in vitro* and *in vivo* from strain CW117 indicated that amidohydrolase ADH3 showed distinguished degradation activity among the identified OTA-detoxify enzymes. As compared to previous results, the *K*<sub>cat</sub>/*K*<sub>m</sub> value of rADH3 (30,3938 s<sup>-1</sup> · mM<sup>-1</sup>) was about 56 (53,63 s<sup>-1</sup> · mM<sup>-1</sup>) and 210 (14,44 s<sup>-1</sup> · mM<sup>-1</sup>) times higher than those of the previously recognized most efficient detoxifying enzymes, rAfOTase and rOTase (11, 15, 16).

Based on an enzymatic activity assay *in vitro*, the *K*<sub>cat</sub>/*K*<sub>m</sub> value of rADH3 was 29,113 times higher than that of detoxifying rNA, indicating the NA contribution to OTA degradation in strain CW117 might be neglected. However, this is not the case for the NA role on OTA degradation *in vivo*. When gene *na* was knocked out from wild-type CW117, the Δna mutant reduced OTA degradation activity in the first 6 h (Fig. 5B). That is, *na* might contribute to OTA degradation in strain CW117 *in vivo* significantly (at least for the first 6 h). In addition, physiological analysis showed that gene *na* knockout did not influence the growth character and ROS level of the strain, and this result indicated that OTA degradation reduction in the Δna mutant could not be ascribed to physiological changes of the CW117. From the RNA expression levels and ADH3 protein contents between wild-type CW117 and the Δna mutant, we found that although *na* knockout did not reduce *adh3* expression level in the Δna mutant at the 6th hour during degradation, the isoenzyme NA can improve the stability of ADH3 in CW117. This result indicated that NA might act as a chaperone that assists the folding of ADH3.

![Figure 6](image-url)
or improves ADH3 stability by other unknown mechanisms. When the gene na was deleted, the stability of the ADH3 protein reduced, and the ADH3 protein content in the Δna mutant at the 6th hour was lower than that of CW117 (Fig. 6B) and, thus, resulted in OTA degradation reduction.

This study further supported that microbial strains show various mechanisms on OTA detoxification. As discussed above, microbial strains can degrade OTA at least through three possible pathways and produce different degradation products (8–11), and some microbial strains (e.g., lactic acid bacteria and yeast) even detoxify OTA by cell-wall adsorption (23–24). Several studies found that the activity of identified OTA-detoxifying enzyme was much lower than that of the host strain, such as, host strain Acinetobacter sp. neg1 and enzyme PJ_1540 (17), strain Bacillus amyloliquefaciens ASAG1 and enzyme rCP (18), strain Lysobacter sp. CW239 and enzyme CP4 (9, 20), and Bacillus subtilis CW14 and enzyme DacA (19, 25). The degradation characteristics of the strains shown above might share similar degradation modes as strain CW117, which contains multiple genes for joint degradation, but the exact degradation mechanisms in these degradation strains are rarely focused. Here, two degradation genes were screened from strain CW117, and two genes showed the interactive effect on the pollutant degradation. To the best of our knowledge, this is the first study that clearly illustrated a degradation mechanism on OTA by multiple detoxifying enzymes in a bacterial strain. However, as discussed above, different microbial strains degraded OTA through different pathways and produced various degraded products, indicating different degradation mechanisms. Even though a strain contains multiple degradation enzymes as in this study, the joint degradation mechanism could be different from the synergistic effect seen in strain CW117; other joint mechanisms (e.g., additive effect, antagonism, or potentiation) may exist. Much effort is still needed to investigate the degradation mechanisms on these degradation strains and detoxifying enzymes that have been identified in the past decade. It will be of great significance to pay attention to the investigation of new mechanisms and the screening of more efficient detoxifying enzymes, which are the substantial bases of detoxification development.

**MATERIALS AND METHODS**

**Strains, media, and plasmids.** Stenotrophomonas sp. CW117 was isolated and preserved in our laboratory (26), Δna and Δna-adh3 mutants and (Δna-adh3)/na and (Δna-adh3)/adh3 complementary strains were constructed in this study. Escherichia coli Trans1-T1 and Escherichia coli BL21 were purchased from TransGen Biotech (Beijing, China). Strain CW117, gene mutants, and complementary strains were cultured in nutrient agar (or nutrient broth) and the E. coli strains were cultured in Luria-Bertani (LB) (Difco, KS, USA) as described previously (9). OTA standard (98.0% purity) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and OTA standard (99.0% purity) was obtained from Romer Labs (Washington, MO, USA). Other strains, plasmids, and the PCR primers used in this study are shown in Table 1.

**Gene cloning, protein expression, and enzymatic characterization.** The primer pairs of adh3-F/R for gene adh3 and of na-F/R for gene na as in Table 1 were used for PCR amplification. Restricted sites of BamHI and XhoI were introduced to the 5’ end of the forward and reverse primers of both genes, respectively. Two genes were amplified using the genomic DNA (gDNA) of CW117, and each gene primer pair with PrimeStar max DNA polymerase (TaKaRa, Dalian, China). PCR conditions for gene adh3 were denatured for 5 min at 98°C followed by 30 cycles of denaturing for 5 s at 98°C, 10 s of annealing at 58°C, and a final 5-min extension at 72°C. PCR conditions for gene na were similar to those for adh3, but the annealing temperature was 58°C, and the PCR products were sequenced by Sangon Biotech (Shanghai, China) using an Applied Biosystems DNA sequencer (ABI PRISM 377).

Genes na and adh3 were cloned and ligated to the pGEX-4T-1 vector and finally transformed into E. coli BL21 according to the procedures of Luo et al. (11). The transformant (containing pGEX/adh3 or pGEX/na) was incubated in AmplR LB broth (containing 0.1 mg/mL ampicillin) at 37°C with 160 rpm agitation. Until the culture optical density at 600 nm (OD600) reached 0.6, the expression was induced by 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) with an additional 8 h incubation at 16°C. After expression, bacterial cells were collected by centrifugation at 8,000 × g for 10 min at 4°C, washed twice with phosphate buffer saline (PBS) (pH 7.2), and disrupted by an ultrasonicator (Qsonica Q700; Qsonica Sonicators, Newtown, USA). The recombinant proteins of RAdH3 or rNA were purified by GSTrap FF columns (GE Healthcare, MA, USA) according to manufacturer’s instructions. The purity of obtained RAdH3 or rNA protein was evaluated by SDS-PAGE (Bio-Rad, Hercules, USA), and the protein concentration was determined using the bicinchoninic acid (BCA) method (27).

Unless otherwise noted, the rNA solution used in this study was prepared in PBS (pH 7.2) at a concentration of 1.0 mg/mL protein. The degradation mixture for rNA contained 200 μL purified rNA and
1.8 mL PBS (pH 7.2) diluted OTA standard at a final concentration of 25 μg/L, and the degradation mixture was incubated at 42°C for 6 to 24 h. As a parallel test, 200 μL PBS instead of rNA solution was used as a control. OTA degradation activity of rADH3 was determined as in Luo et al. (11). After degradation, OTA residues were examined by an HPLC apparatus (Waters 2695; Waters, Milford, MA, USA) that was equipped with a fluorescence detector (excitation wavelength [λex] = 333 nm; emission wavelength [λem] = 460 nm). OTA and the degradation product were separated by a C18 column (250 mm/C24.6 mm, 5 μm; Xbridge, Milford, MA USA). The mobile phase contained acetonitrile/water/acetic acid (48:51:1, vol/vol/vol), the flow rate was 1.0 mL/min, and the injection volume was 5.0 μL. OTA-degraded product was purified and identified by LC-MS/MS following the method previously described (9).

The optimal temperature was evaluated by 200 μL rNA mixed with 1.8 mL PBS (pH 7.2) diluted OTA standard (final concentration, 25 μg/L) and incubated for 12 h at 0, 10, 20, 30, 37, 40, 42, 45, 48, and 50 to 100°C with intervals of 10°C. The temperature test on rADH3 was described in Luo et al. (11).

| Strain, plasmid, or primer | Characterization or oligonucleotide sequencea,b | Reference/source or endonuclease/primer description |
|----------------------------|-------------------------------------------------|---------------------------------------------------|
| Strain                     |                                                  |                                                   |
| CW117                      | Wild-type degradation strain                     | This study                                        |
| pGEX/na                    | na expression strain                             | This study                                        |
| pGEX/adh3                  | adh3 expression strain                           | This study                                        |
| ∆na                       | Gene na mutant of CW117                          | This study                                        |
| ∆na-adh3                   | Double gene (na and adh3) mutant of CW117        | This study                                        |
| (na-adh3)/adh3             | Gene na complementary for ∆na-adh3 (GmR)        | This study                                        |
| (na-adh3)/adh3             | Gene adh3 complementary for ∆na-adh3 (GmR)      | This study                                        |
| Escherichia coli Trans1-T1 | Gene receptive host                              | TransGen Biotech, Beijing, China                  |
| Escherichia coli BL21      | Gene expression host                             | TransGen Biotech, Beijing, China                  |
| Plasmid                    |                                                  |                                                   |
| pK18mobsacB                | Allelic exchange vector (KmR)                    | 31, 32                                            |
| pSRK-Gm                    | Gene complementary vector (GmR)                  | 34                                                |
| pGEX-4T-1                  | Gene expression vector (AmpR)                    | Geneland, Shanghai, China                         |
| pMD18-T                    | Gene sequencing vector (AmpR)                    | TransGen Biotech, Beijing, China                  |
| Primer                     |                                                  |                                                   |
| na-F                       | CGCGGATCCATGTCGAGGGCGCCCGCGCGGT                 | BamHI                                             |
| na-R                       | CCCTCGAATTCAGTCGAGGGCGCCCGCGCGGT                 | Xhol                                              |
| adh3-F                     | CGGGATCCATGTCGAGGGCGCCCGCGCGGT                  | BamHI                                             |
| adh3-R                     | CCCTCGAATTCAGTCGAGGGCGCCCGCGCGGT                 | Xhol                                              |
| no-US-F                    | TGGTCTAGAGAGAGATGCGGGGTAGGTG                   | Xbal                                              |
| no-UR-R                    | CCGGCAATTCGTCGAGGGCGCCCGCGGT                   |                                                   |
| no-DS-F                    | tggcagcagaagagcttggtCCCGCGCGCGCGGT              | Lowercase indicates sequence for overlap PCR      |
| no-DS-R                    | CCGGACGCAGGCAATGCGCGCGCGGCGGT                   | HindIII                                           |
| val1-F                     | GTCAACTGTCGAGGGCGCCCGCGGT                      | na mutant verify                                  |
| val1-R                     | GCCTTTTTTGTATGCGGT                             |                                                   |
| com-na-F                   | GGAATTTCATATAGTAAGTGCAGAGAGAGCTTCTTG            | Ndel                                              |
| com-na-R                   | CCGGACGCAGGCAATGCGCGCGCGGCGGT                   | HindIII                                           |
| val2-F                     | TGCGTGTCGTTCGAGGGCGCGGT                        | adh3 mutant verify                                |
| val2-R                     | TCCCTGCTGTCGAGGGCGCGGT                        |                                                   |
| adh3-US-F                  | GCTCTAGATATGAGGGCGAGATGCGGT                   | Xba I                                             |
| adh3-US-R                  | GCACGCTGTCGAGGGCGAGATGCGGT                    |                                                   |
| adh3-DS-F                  | aatacctgctgacgctgATCTACGAAGTACGTAGGATT         | Lowercase indicates sequence for overlap PCR      |
| adh3-DS-R                  | CCGGACGCAGGCAATGCGCGCGGCGGT                   | HindIII                                           |
| val3-F                     | GGCGATTCGAGGGCGCCCGGT                      | na complementary verify                          |
| val3-R                     | ATACGCAACGGCTCCTC                              |                                                   |
| com-adh3-F                 | GGAATTTCTATGATAATGCAGAGAGAGCTTCTTG             | Ndel                                              |
| com-adh3-R                 | CCGGACGCAGGCAATGCGCGCGCGGCGGT                   | Nhel                                              |
| val4-F                     | CACCGAGGAGGGCGAGGGCGGT                      | adh3 complementary verify                        |
| val4-R                     | CACCGAGGAGGGCGAGGGCGGT                      |                                                   |
| F gapdh                    | CCGGACGAGGGCGAGGGCGGT                      | Reference gene gapdh RT-qPCR primer               |
| R gapdh                    | TGGAGCGAGGCAAGACGAC                         | Reference gene gapdh RT-qPCR primer               |
| F na                       | ACCGGCACCCGGCAACGAC                        | Gene na RT-qPCR primer                           |
| R na                       | GGAGCCGACGCCAAGAGACAC                      | Gene na RT-qPCR primer                           |
| F adh3                     | CAAGTCCATCCGACGAC                         | Gene adh3 RT-qPCR primer                          |
| R adh3                     | ACCGAGTGGACCCGACGAC                        | Gene adh3 RT-qPCR primer                          |

aRestriction sites are underlined. Lower cases indicate sequence for overlap PCR.
bGmR, gentamicin resistance; KmR, kanamycin resistance; AmpR, ampicillin resistance.
optimal pH (the buffer for pH 2 to 5 was glycine-HCl, the buffer for pH 6 to 7 was PBS, the buffer for pH 8 to 9 was Tris-HCl, and the buffer for pH 10 to 11 was glycine-NaOH), the effects of metal ions (i.e., Cu\(^{2+}\), Zn\(^{2+}\), Fe\(^{3+}\), Ca\(^{2+}\), Li\(^{+}\), Mg\(^{2+}\)), the effects of metal chelators (i.e., EDTA and EGTA), and the effects of protein denaturants (SDS or proteinase K, or SDS plus proteinase K) on the two detoxifying enzymes were performed as previously described (9). In each enzymatic test, 200 \(\mu\)L of enzyme solution (i.e., 0.2 mg protein) or 2.4 \(\mu\)L of ADH3 (i.e., 1.2 \(\mu\)g protein) was used in a 2-ml mixture; the incubation time for \(\text{ADH3}\) was 12 to 48 h, and the incubation time for rADH3 was 2 min.

In a kinetic constant test, 500 \(\mu\)L of enzyme solution (i.e., 0.25 mg protein) was mixed with 500 \(\mu\)L of OTA solution with different concentrations (15 \(\mu\)g/L, 25 \(\mu\)g/L, 35 \(\mu\)g/L, 45 \(\mu\)g/L, 55 \(\mu\)g/L, and 65 \(\mu\)g/L). The degradation product OTo was determined by HPLC after reacting at 42°C for 6 h. The same volume of PBS (pH 7.2) instead of \(\text{OTA}\) solution was used as the control. The kinetic constants of \(K_m\), \(K_{in}\), and \(V_{max}\) were calculated following the methods as previously described by a nonlinear regression of Michaelis-Menten equation (11, 28).

**Phylogenetic analysis on detoxifying enzymes and microbial hosts.** The OTA-detoxifying enzymes of this study and those from other peer-reviewed publications with known gene sequences were retrieved from the GenBank database for phylogenetic analysis (11). After multiple sequence alignment by Clustal X 1.8 (29), a phylogenetic tree on the detoxifying enzymes was constructed using MEGA 7.0 (30). Kimura’s two-parameter model was selected to calculate the corrected evolutionary distance, and the neighbor-joining (NJ) algorithm was used for clustering (31-32). Bootstrap analysis was applied to determine tree topology by 1,000 resamplings (33). The potential hosts (e.g., bacteria, fungi strains and animals) of each detoxifying enzyme were obtained by BLAST searches using the gene sequences of detoxifying enzymes. The housekeeping genes of the hosts were retrieved from the GenBank database, and phylogenetic analysis on the hosts were constructed following the same method as detoxifying enzymes.

**Polyclonal antibody preparation and Western blotting.** Recombinant protein ADH3 produced from *E. coli* BL21 was purified and used for polyclonal antibody production as discussed previously (34). New Zealand White rabbits are immunized by subcutaneous injection of rADH3 (400 \(\mu\)g), which is emulsified in Freund’s complete adjuvant, and are boosted every 3 weeks. Serum was collected 10 to 14 days after the last injection and assayed for antibody activity by the indirect enzyme-linked immunosorbent assay (ELISA) method. The antiserum was collected for further affinity purification using rADH3-conjugated agarose beads. The purified antiserum was quantified and verified by Western blotting.

In ADH3 Western blotting, CW117 cells were washed twice with PBS, suspended in lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.1% 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate [CHAPS], 1 mM EDTA, 1 mM NaF, 1 mM NaVO\(_3\), and protease inhibitors), and disrupted by sonication on ice as above. Protein concentration was determined by the BCA method (27). Ten micrograms (10 \(\mu\)g) of protein were used per well in a 5-ml sample; the incubation time for rNA was 12 to 24 h. The Western blot was performed using an antibody to ADH3 at 1:1,000 dilution. The antibody was quantified and verified by Western blotting.

**Gene mutant construction.** Recombinant plasmid pK18mobsacB_US-DS\(_{sacB}\) (or pK18mobsacB_US-DS\(_{sacB}\)) was constructed for gene mutation as shown in Fig. 5A in the supplemental material, and the scheme of mutants screening is shown as in Fig. 5B. The gene knock-out procedures followed previously published methods (36-37). Usually, the upstream (US) and downstream (DS) DNA fragments that were adjacent to gene \(\text{no}\) were amplified by PCR using the PrimerSTAR Max DNA polymerase (Takara, Dalian, China). Two primer pairs, \(\text{no-US}\)-F and \(\text{no-US}\)-R as well as \(\text{no-DS}\)-F and \(\text{no-DS}\)-R, were used for \(\text{no-US}\) and \(\text{no-DS}\) amplification, respectively (Table 1). The PCR conditions for \(\text{no-US}\) (or \(\text{no-DS}\)) were as follows: 10 min denaturing at 98°C followed by 30 cycles of 10 s denaturing at 98°C, 5 s annealing at 61°C, 10 s extension at 72°C, and a final 10 min extension at 72°C. PCR product of \(\text{US-DS}\) was purified and digested with XbaI and HindIII at 37°C overnight. The digest solution consisted of 1.0 \(\mu\)L HindIII, 1.0 \(\mu\)L XbaI, 0.0 \(\mu\)L US-DS\(_{sacB}\) (or 1 \(\mu\)g pK18mobsacB), 5 \(\mu\)L 10× M buffer (Takara, Dalian, China), and supplemented with double-distilled water (ddH\(_2\)O) to 50 \(\mu\)L. Digested US-DS\(_{sacB}\) was ligated to suicide plasmid pK18mobsacB, which had been digested with the same restriction enzymes, XbaI and HindIII. The ligation solution contained 4 \(\mu\)L digested US-DS\(_{sacB}\), 2 \(\mu\)L ligated pK18mobsacB, and 6 \(\mu\)L solution I DNA ligase, and the ligation was incubated at 16°C for 1 h. After that, 5 \(\mu\)L ligated product was transformed to *E. coli* Trans1-T1 and screened by LB agar with KanR (i.e., 50 \(\mu\)g/ml kanamycin). Positive clones were identified by PCR sequencing, and the correct recombinant plasmid was defined as pK18mobsacB_US-DS\(_{sacB}\).

The clone containing the recombinant plasmid was enriched by KanR LB broth and extracted by a Plasmid MiniPrep kit (Axygen, CA, USA). Plasmid pK18mobsacB_US-DS\(_{sacB}\) was transformed into wild-type CW117 by the electroporation method as in Sheng et al. (38). Transformed cells were spread on the nutrient agar with KanR. A single colony on KanR screening agar was transferred to 5 mL KanR nutrient broth, incubated at 37°C with an agitation of 160 rpm. Until the OD\(_{600}\) reached 0.8, bacterial cells were centrifuged at 8,000 \(\times\) \(g\) and washed twice with sterile nutrient broth and resuspended.
in nutrient broth. Serial nutrient broth-diluted cells were spread on nutrient agar containing 12% sucrose (i.e., “sucrose screening agar”), and the clones grown on sucrose screening agar were re-screened by KanR nutrient agar. The resulting colonies that can grow on sucrose screening agar but are susceptible to kanamycin are considered to be the Δna mutant candidate. Mutant candidate was verified by PCR sequencing with PrimerSTAR Max DNA polymerase and by verify primers val1-F and val1-R (Table 1). The PCR conditions were as follows: 5 min denaturing at 98°C followed by 30 cycles of 10 s denaturing at 98°C, 5 s annealing at 59°C, 10 s extension at 72°C, and a final 10 min extension at 72°C.

The Δadh3 mutant was constructed and screened by the same protocol of Δna. For double gene mutant construction and screening, gene adh3 was further deleted from the Δna mutant genome by the same protocol as gene na knockout from wild-type CW117, producing the Δna-adh3 mutant. The PCR primers ΔSna-ms (adh3-US-F, adh3-US-R), ΔSna-m (adh3-DS-F, adh3-DS-R), and adh3 knockout verify fragment (val2-F and val2-R) are shown in Table 1.

**Gene complementary strain construction.** Gene na or adh3 was cloned to vector pSRK-Gm to produce gene recombinant plasmid pSRKGm/na or pSRKGm/adh3 according to the method of Khan et al. (39). The complementary plasmid (pSRKGm/na or pSRKGm/adh3) was transformed into the cells of the Δna-adh3 double mutant gene by electroporation method as previously described to obtain the complementary strain (Δna-adh3)/na or (Δna-adh3)/adh3. As shown in Fig. S6 in the supplemental material, the na open reading frame (ORF) was amplified by PCR using the PrimerSTAR Max DNA polymerase and primers com-na-F and com-na-R (Table 1). PCR conditions were as follows: 5 min denaturing at 98°C followed by 30 cycles of 10 s denaturing at 98°C, 10 s annealing at 65°C, 20 s extension at 72°C, and a final 5 min extension at 72°C. Purified na PCR product and vector pSRK-Gm (gentamicin resistance [GmR]) were double digested by HindIII and NdeI at 37°C overnight, respectively. Digest mixture consisted of 1.0 μL HindIII, 1.0 μL NdeI, 10.0 μL na PCR product (or 1 μg pSRK-Gm), 5 μL 10× M buffer, and supplement L HindIII, 1.0 μL NdeI, and 10.0 μL pSRK-Gm. Digested na PCR product was ligated to vector pSRK-Gm, which had been digested with the same restriction enzymes. The ligation mixture contained 2 μL digested pSRK-Gm (GmR), 4 μL digested na, and 6 μL solution 1 DNA ligase, and the ligation was incubated at 16°C for 1 h. After that, 5 μL ligated product was transformed to E. coli Trans1-T1 and spread by LB agar with 50 μg/mL gentamicin (GmR). Transformant was enriched by LB broth (GmR), and the plasmid pSRK-Gm/na (GmR) was extracted using a plasmid miniprep kit. Recombinant plasmid pSRK-Gm/na (GmR) was validated and was transformed into the Δna-adh3 mutant by electroporation. After that, transformant was spread on GmR nutrient agar. Grown colonies on GmR nutrient agar were the complementary candidate (Δna/adh3)/na. The candidate was verified by PCR sequencing with PrimerSTAR Max DNA polymerase, and the primers of val3-F and val3-R. The PCR conditions were the same as the gene na clone from the CW117 genome.

The adh3 complementary strain, (Δna-adh3)/adh3 strain, construction followed the same procedures as gene na. The PCR primers of gene adh3 were com-adh3-F and com-adh3-R, and PCR primers for adh3 complementary strain verification were val4-F and val4-R (Table 1).

**OTA degradation tests on mutants and complementary strains.** Before degradation tests, wild-type CW117, mutants, and complementary strains were further verified by PCR sequencing. Meanwhile, the adh3 expression levels in wild-type CW117, the Δna-adh3 double mutant, and the (Δna-adh3)/adh3 complementary strain were examined by Western blotting during OTA degradation at the 12th hour.

In mutant degradation tests, strain CW117 and mutants were inoculated into nutrient broth with a final OTA concentration of 50 μg/L. The degradation tests were performed as in Wei et al. (9), and samples were collected at hours 0, 3, 6, and 9 for OTA residue analysis. In complementar strain degradation tests, wild-type CW117 and the Δna-adh3 double mutant were used as controls. The control and complementary strains were inoculated into nutrient broth with a final OTA concentration of 50 μg/L. The degradation tests were performed as previously described, but 0.1 mM IPTG was added to the complementary strains to induce complementary gene expression (39). Samples were collected at hours 0, 3, 6, 9, and 12 for OTA residue analysis.

**Growth curves and reactive oxygen species determination.** Growth curves of CW117 and mutants were determined by fresh bacterial culture according to the method of Qian et al. (20). For ROS evaluation, fresh culture of wild-type CW117, the Δna mutant, or the Δna-adh3 double mutant was inoculated to nutrient broth with different OTA contents (50 and 300 μg/L), and the cultures were collected at 6th and 12th hours for ROS determination. Bacterial cells (1 mL) were collected and further incubated with 1.0 mL (10 μM) 2′,7′-dichlorodihydroflourescein diacetate (H2-DCFDA) (BioSharp, Beijing China) for 30 min at 37°C. After incubation, the bacterial cells were washed twice with ultrapure water and resuspended in 1 mL PBS (pH 7.2) for the following fluorescence examination. As a positive control, the bacterial cells pretreated in 1.0 mL Rosup reagent (BioSharp, Beijing China) for 1 h at 37°C were collected by centrifugation and used for the following H2-DCFDA incubation and fluorescence examination. As the negative control, the bacterial cells were washed twice with ultrapure water and resuspended in 1.0 mL PBS (pH 7.2) for H2-DCFDA fluorescence examination. H2-DCFDA fluorescence was examined by flow cytometry (CytoFLEX; Beckman Coulter, CA, USA) following the procedures as previously described (21).

**Degradation gene expressions during OTA degradation.** For joint degradation evaluation, gene expressions (including na and adh3) were determined and compared at the 6th hour between wild-type CW117 and the Δna mutant. Digested during the OTA degradation process. In addition, bacterial strain CW117 under the mid-log phase was induced by 50 and 100 μg/L substrate OTA for 40 min, respectively, and collected for two genes’ OTA induction character examination. RNA was extracted by RNAprep pure bacteria kit (TransGen, Beijing, China), and reverse transcription was performed by FastKing RT kit (TransGen, Beijing, China) according to the manufacturer’s instructions. Gene expression levels were examined by reverse-transcription quantitative PCR (RT-qPCR) using SuperReal PreMix Plus (SYBR green)
from TransGen Biotech (Beijing, China) following the protocols of Rocha et al. (40). The primer pairs F
R
and F
R
were used for the amplification of two genes, and the primers of F
and R
were used for gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as an internal reference (Table 1). PCR conditions were as follows: 2 min denaturing at 95°C followed by 39 cycles of 5 s denaturing at 95°C, 30 s annealing, and extension at 60°C, and continued for 1 cycle of 5 s denaturing at 95°C, 5 s annealing, and extension at 65°C. Other than RNA examination by RT-qPCR, the expression levels of superefficient enzyme ADH3 in CW117 or the Δm mutant were further determined by Western blotting.

**Statistical analysis.** Unless otherwise noted, the assays in this study were performed in triplicate. Analysis data are shown as the mean ± standard deviation (SD). Student’s t test was selected for statistical analysis. Significant difference was accepted at a P value of <0.05.

### SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.**

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We declare no conflict of interest.

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### Interactive Effect of Two Isoenzymes of Amidohydrolase

**Microbiology Spectrum**

July/August 2022 Volume 10 Issue 4

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