Substrate Specificity and Colorimetric Assay for Recombinant TrzN Derived from *Arthrobacter aurescens* TC1

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The TrzN protein, which is involved in *s*-triazine herbicide catabolism by *Arthrobacter aurescens* TC1, was cloned and expressed in *Escherichia coli* as a His-tagged protein. The recombinant protein was purified via nickel column chromatography. The purified TrzN protein was tested with 31 *s*-triazine and pyrimidine ring compounds; 22 of the tested compounds were substrates. TrzN showed high activity with sulfur-substituted *s*-triazines and the highest activity with ametryn sulfoxide. Hydrolysis of ametryn sulfoxide by TrzN, both in vitro and in vivo, yielded a product(s) that reacted with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) to generate a diagnostic blue product. Atrazine chlorohydrolase, AtzA, did not hydrolyze ametryn sulfoxide, and no color was formed by amending those enzyme incubations with NBD-Cl. TrzN and AtzA could also be distinguished by reaction with ametryn. TrzN, but not AtzA, hydrolyzed ametryn to methylmercaptan. Methylmercaptan reacted with NBD-Cl to produce a diagnostic yellow product having an absorption maximum at 420 nm. The yellow color with ametryn was shown to selectively demonstrate the presence of TrzN, but not AtzA or other enzymes, in whole microbial cells. The present study was the first to purify an active TrzN protein in recombinant form and develop a colorimetric test for determining TrzN activity, and it significantly extends the known substrate range for TrzN.

The *s*-triazine ring is found in pesticides and dyes, which are increasingly being found to be biodegradable in the environment. The biodegradation of *s*-triazine herbicides has been demonstrated in diverse genera of bacteria distributed globally, but bacterial isolates are often found to have enzymes with nearly identical amino acid sequences (1, 15, 26). The enzymes elaborated for metabolism of *s*-triazine herbicides have been most widely studied in *Pseudomonas* sp. strain ADP (10, 28). *Pseudomonas* sp. strain ADP typifies atrazine metabolism by gram-negative organisms studied to date, which have been shown to contain genes with very high sequence identity to *atzA*, *atzB*, and *atzC*. The *atzA*, *-B*, and *-C* genes encode enzymes that hydrolytically and consecutively remove each of the three substituents on the 1,3,5-triazine ring carbon atoms to generate cyanuric acid. The enzyme initiating *s*-triazine herbicide metabolism in *Pseudomonas* sp. strain ADP is AtzA, atrazine chlorohydrolase. AtzA has been purified and characterized (3). AtzA was shown to have a very narrow substrate specificity and is only active with fluoroatrazine and 2-chloro-4,6-N-monoalkyl- or 2-chloro-4,6-N-dialkyl-1,3,5-triazine compounds (18). DNA shuffling of the *atzA* gene with the *triA* gene encoding melamine deaminase generated hybrid enzymes active on a much wider range of *s*-triazine substrates (14).

More recently, a number of gram-positive bacteria have been observed to metabolize a broader range of *s*-triazine herbicides than the gram-negative bacteria (15, 20, 23, 27).

Among these are *Nocardioides* sp. strain C190, which did not contain genes with close sequence identity to *atzA*, *atzB*, or *atzC*. Topp and coworkers (27) prepared crude extracts from *Nocardioides* sp. strain C190, purified the enzyme that transformed atrazine to hydroxyatrazine, and called the protein TrzN. TrzN was tested with five substrates and was most active with the sulfur-substituted *s*-triazine herbicides ametryn and prometryn. The products of the reaction with the different herbicides are hydroxy-*s*-triazines that are substrates for AtzB and AtzC (28). Some of the gram-positive bacteria containing the *trzN* gene have also been shown to contain *atzB* and *atzC* (13, 16). The *trzN* gene from *Nocardioides* sp. strain C190 has been cloned into *Escherichia coli*, sequenced, and found to have homology to AtzA (12), thus making it a member of the amidohydrolase superfamily (8). However, TrzN is significantly divergent from AtzA, with which it shares only 26% sequence identity at the amino acid level (12). Efforts to express TrzN activity in *E. coli* were unsuccessful. Most recently, a *trzN* gene was found on a 160-kb plasmid-borne gene region in *Arthrobacter aurescens* TC1 (16).

In the present study, the *trzN* gene from *Arthrobacter aurescens* TC1 was cloned, expressed as a His-tag protein in *E. coli*, and purified to homogeneity. TrzN, but not AtzA, was active with ametryn and ametryn sulfoxide, and this provided the basis for a selective colorimetric assay for TrzN in vitro. In vivo analyses indicated that only bacteria expressing TrzN were shown to develop color, showing the utility and selectivity of the assay.

MATERIALS AND METHODS

Chemical sources and syntheses. All buffers and routine biochemicals were obtained from Sigma-Aldrich (St. Louis, MO) and were of the highest available

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purity. 7-Chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) was from Sigma-Aldrich. Syngenta (Greensboro, NC) graciously provided the following: atrazine (CIET), simazine (CEET), terbutylazine [CE(tB)T], ametryn [(Ms)IET], prometryn [(Ms)IIT], deethylatrazine (CIAT), and deisopropylatrazine (CEAT). 2-Chloro-4,6-diamino-1,3,5-s-triazine (CAAT) was purchased from Sigma-Aldrich. All other compounds were synthesized in our laboratory. Fluoroatrazine (FIET), azidoatrazine [(N₃)IET], cyanoatrazine [(CN)IET], atratone [(Mo)IET], aminoatrazine [(NH₂)IET], 2-chloro-di(*N*-ethylamino)-1,3-pyrimidine (CEEP), and 2,6-di(*N*-ethylamino)-4-chloro-1,3-pyrimidine were synthesized as previously described (18). All diaminochloro-*s*-triazines were prepared as previously described by Thurston et al. (25). Alkoxy-*s*-triazines were prepared from the alcohols and sodium bicarbonate as previously described by Dudley et al. (4). 2-Methylthio-4,6-diamino-1,3,5-triazine [(Ms)AAT] was prepared by heating 2-chloro-4,6-diamino-1,3,5-triazine with equimolar amounts of sodium hydrosulfide and methylmercaptan disulfide at 90°C in water. Potassium bromide was added as a catalyst, and sodium carbonate was added to neutralize acid. After 1 h, the reaction was cooled and filtered. The solid was retained, and the filtrate was combined with 100 g NaCl and 100 ml ethyl acetate and stirred to dissolve the salt. After separation, the aqueous layer was extracted twice more with 100-ml portions of ethyl acetate. The organics were dried over MgSO₄, combined with 300 ml methanol and the retained solids, and heated to boiling. Filtration and cooling of the liquid yielded solid 2-methylthio-4,6-diamino-1,3,5-triazine.

2-Mercapto-4-isopropylamino-6-ethylamino-1,3,5-triazine (SIET) was prepared by refluxing atrazine, thiourea, and water in ethanol under nitrogen conditions. After refluxing for 14 h, the reaction mixture was cooled, sodium bicarbonate was added, and the mixture was stirred and shaken until gas evolution ceased and then filtered. The material in the filter cake, 2-mercapto-4-isopropylamino-6-ethylamino-1,3,5-triazine, was recrystallized in hot ethanol under a nitrogen atmosphere and dried under vacuum conditions. 2-Isopropylamino-4-ethylamino-1,3,5-triazine [IE(HT)] was prepared by mixing atrazine–5% Pd on CaCO₃ and Na₂CO₃ in isopropanol under hydrogen gas at 25°C. The reaction was monitored daily by gas chromatography (GC) using a flame ionization detector until the remaining atrazine was less than 1% of the starting material. The reaction mixture was heated to dissolve the product and filtered, and the collected solid was extracted with hot ethanol. Filtrates were evaporated on the rotary evaporator, dissolved in ethyl acetate, and passed through silica gel and Celite columns to remove finely divided palladium. The 2-isopropylamino-4-ethylamino-1,3,5-triazine product was recrystallized twice from ethyl acetate to remove residual atrazine. The product was found to be 99.1% pure by gas chromatography-mass spectrometry (GC-MS). Ametryn sulfoxide was synthesized by adding, slowly over 30 min, 1.5 equivalents of *m*-chloroperoxybenzoic acid to ametryn in chloroform under nitrogen at 5°C. After an additional 30 min, the reaction mixture was washed twice with saturated NaHCO₃, dried over MgSO₄, and filtered through silica gel. Contaminants of ametryn and a small amount of the sulfone were removed by repeated chromatography on silica gel with dichloromethane as a solvent and slow crystallization of the product from dichloromethane.

Gene cloning and expression. Total genomic DNA was extracted from cell pellets of *Arthrobacter aureus* TC1 as follows: cells were resuspended in 100 ml Tris-HCl, pH 8.0, containing 0.5 M sucrose, 100 mg per ml lysozyme, and 6.25 mM EDTA. The cell suspension was incubated for 4 h at 37°C. Cells were subjected to freeze-thawing, suspended in 0.2 mg/ml proteinase K–0.5% sodium dodecyl sulfate (SDS)–0.8 M NaCl–1% cetyltrimethylammonium bromide, and incubated for 10 min at 65°C and then overnight at 4°C. Precipitated DNA was purified on a CsCl gradient. The *trzN* gene from *A. aureus* TC1 (accession number AAS20185) was amplified, without its native promoter, by PCR using primers 5'-GCCATATGATCCTGATCCG-3' and 5'-GCAAGCTTCTACAAGTTCTTGG-3'; the primers contained *Nde*I and *Hind*III restriction enzyme sites followed by a GC addition at their 5' ends, respectively. The gene was cloned downstream of a T7 promoter and an N-terminal six-His-tag clamp in the vectors *pET29* and *pET28b+* (Novagene, Madison, WI). The constructs were transformed into *E. coli* BRL21(DE3), and their sequences were verified. *E. coli* BRL21(DE3) (*pET28b+::trzN*) was grown in Luria-Bertani medium (17) containing 50 µg kanamycin per ml at 25°C, with shaking at 150 rpm. When culture reached an attenuation of 0.5 at 600 nm, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and the induced cells were grown for 8 h under the same conditions.

Cell growth. *Arthrobacter aureus* TC1 (23), *Nocardioide*s sp. strain C190 (27), *Pseudomonas* sp. strain ADP (9), *Agrobacterium radiobacter* J14a (24), and *Alcaligenes* sp. strain SG1 (18) were grown in R minimal medium (5) containing 10 mM glucose and 6.8 mM sodium citrate as carbon sources and 463 µM

atrazine as the sole nitrogen source. Cultures were incubated at 30°C, with shaking, until a maximum optical density at 600 nm was observed.

Enzyme purification. For enzyme purification, 5 liters of *E. coli* BRL21(DE3) (*pET28b+::trzN*) cells were grown as described above. The cells were washed three times with 0.85% NaCl solution and centrifuged at 6,000 × *g*, and the pellet was resuspended in 50 ml of 50 mM potassium phosphate buffer, pH 7.0. The cell suspension was passed three times through a chilled French pressure cell operated at 140 MPa, and the crude cell lysates were cleared by centrifugation at 18,000 × *g* for 90 min at 4°C. TrzN was purified using a 5 ml HiTrap chelating HP column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and a Pharmacia FPLC LKB system (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The column was reequilibrated with 2.5 ml 0.1 M NiCl₂ before every use, washed with 15 ml of distilled water, and equilibrated with 50 ml of 25 mM MOPS (morpholinepropanesulfonic acid), pH 7.0, at a flow rate of 1 ml/min. Protein (approximately 300 mg) was injected manually via a Pharmacia super loop (50 ml capacity) onto the column at a flow rate of 1 ml/min. The column was washed with 25 mM MOPS, pH 7.0. Protein was eluted from the column with a step gradient consisting of 0.05 M, 0.25 M, and 0.5 M imidazole in 25 mM MOPS, pH 7.0, at a flow rate of 2 ml/min. Fractions (2 ml) were collected throughout the steps and tested for TrzN activity. Purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, Hercules, CA).

Enzyme assay. Enzyme activity was measured by monitoring the decrease in absorbance of ametryn for 10 min at 264 nm by using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). The product hydroxyatrazine has negligible absorbance at this wavelength. Reactions were carried out in 1 ml 50 mM potassium phosphate buffer, pH 8.0, containing 132 µM ametryn at 25°C. Reactions were initiated by the addition of the enzyme. The molar absorbance for ametryn under these conditions was determined to be 5,000 M⁻¹ cm⁻¹ at 264 nm.

Substrate incubations. Compounds tested as potential substrates were prepared as saturated solutions in 0.1 M potassium phosphate buffer, pH 8.0. Methanol, at concentrations ranging from 0.05 to 10%, was added to each solution to increase the solubility of compounds, which were at final concentrations ranging from 1 to 60 mg per liter. All solutions were filtered through a 0.2-µm Acrodisc CR 13-mm syringe filter (Pall Gelman Laboratory, Ann Arbor, MI) prior to use. TrzN was incubated with each potential substrate at 37°C, and at selected time points subsamples were taken for high-pressure liquid chromatography (HPLC) analysis using a Hewlett-Packard HP 1100 system equipped with a photodiode array detector interfaced to an HP ChemStation. An Adsorbosphere C₁₈ 5µ column (Alltech, Deerfield, Ill.) (250 by 4.6 mm) was used to separate *s*-triazines and pyrimidines with a methanol-water gradient as follows: 5 min at 50% methanol; 10 min linear gradient to 100% methanol; 5 min at 100% methanol; 5 min linear gradient to 50% methanol; and 50% methanol for 2 min. When compounds were shown by HPLC to be highly reactive with TrzN, enzyme assays were conducted spectrophotometrically with 30 µg of each compound per ml as described above. The disappearance of ametryn, atrazine, and ametryn sulfoxide was measured at 264 nm, and depletion of cyanoatrazine was measured at 300 nm.

Colorimetric assay. A colorimetric assay was developed to monitor TrzN activity and to differentiate it from other triazine-transforming enzymes *in vitro* and *in vivo*. Atrazine-degrading bacterial strains, *Arthrobacter aureus* TC1, *Nocardioide*s sp. strain C190, *Pseudomonas* sp. strain ADP, *Agrobacterium radiobacter* J14a, and *Alcaligenes* sp. strain SG1, were grown on R minimal medium plates (5) supplemented with 500 mg per liter atrazine as the sole nitrogen source. Glucose and sodium citrate were the carbon sources. A few colonies from each strain were resuspended in 300 µl of 0.1 M sodium phosphate buffer, pH 8.0, and 20-µl aliquots were incubated with 1 ml of a saturated solution of ametryn sulfoxide in the same buffer. After 1 h incubation at 25°C, 20 µl of 167 mM 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) was added to each assay and color development at 590 nm was monitored using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). *Arthrobacter aureus* TC1 lyophilized cells (5 mg) were analyzed under the same conditions. Controls consisted of reaction mixtures with no bacteria or with no substrate. The enzymes TrzN and AtzA (3), AtzB (J. Osborne, personal communication), and AtzC (19) were purified as described previously. Each purified enzyme (50 µg) was mixed with 1 ml saturated solution of ametryn sulfoxide and 20 µl NBD-Cl and incubated for 3 h, and color development was monitored as described above. The same experiments were conducted using ametryn as the substrate, with color development being monitored at 420 nm.

Product determination. The products of ametryn and ametryn sulfoxide hydrolysis by TrzN, before and after the addition of the colorimetric reagent NBD-Cl, were determined by HPLC and GC-MS using an HP 6890/5973 instru-

FIG. 1. Elution profile of TrzN from the HiTrap chelating HP column with imidazole step gradient. Dashed line, imidazole concentration; solid line, absorbance at 280 nm. Insert: SDS-PAGE of purified TrzN. Lane 1, crude cell extract; lane 2, fraction eluted with 0.25 M imidazole. Numbers on the left correspond to molecular mass in kDa.

ment (Hewlett-Packard, San Fernando, CA). Hydroxyatrazine was detected using HPLC as previously reported (2). For the GC-MS analysis, 1 ml of the enzymatic reaction mixture was extracted with 0.5 ml dichloromethane and dried with sodium sulfate. The headspace of an *A. aureescens* TC1 culture growing with ametryn was sampled using a 10-ml Hamilton gas-tight syringe and analyzed by direct injection into a GS-MS instrument. The products were separated on an HP-5 (cross-linked 5% Me Ph siloxane) column (Agilent Technology, Hewlett-Packard, San Fernando, CA) (30-m length, 0.25- μ m film thickness, and 0.25-mm column inside diameter) with the following temperature gradient: 50°C for 3 min, followed by a 10 min linear gradient to 300°C, and hold at 300°C for 3 min. Helium served as the carrier gas with a constant flow rate of 1 ml per min.

RESULTS AND DISCUSSION

Cloning, expression, and purification of TrzN from *E. coli*. PCR primers for an external region of the *trzN* gene (accession number AAS20185) were used to amplify the gene from *A. aureescens* TC1 total genomic DNA. The resulting PCR product was subsequently cloned into the pET29 vector behind a T7 phage promoter and sequenced.

The pET29::*trzN* construct was transformed into *E. coli* BRL21(DE3), and cell extracts were evaluated for TrzN activity. However, none of the crude extract preparations had the ability to turn over ametryn or atrazine. SDS-PAGE gels revealed that most of the protein was present in the insoluble fraction (data not shown), which is consistent with the formation of inclusion bodies.

The *trzN* gene was subsequently cloned into pET28b+ containing a His tag at the N terminus. Cells containing this construct were lysed, and the soluble crude cell protein fraction contained ametryn hydrolase activity. SDS-PAGE showed a protein band with an apparent molecular mass of 50,000 Da that was not present in native *E. coli*.

The soluble fraction was loaded onto a HiTrap chelating HP column and eluted with an imidazole step gradient (Fig. 1). Tests for ametryn hydrolysis showed that TrzN was eluted at the 0.25 M imidazole fraction. The enzyme was purified 44-fold, yielding 2.8 mg protein with 47% recovery of activity. The specific activity of the protein purified here was comparable to that for the hydrolysis of atrazine by the homolog AtzA (3) and approximately 1 order of magnitude lower than the activity reported for the hydrolysis of ametryn by TrzN from *Nocardioide*s spp. (27).

The predicted recombinant protein sequence, containing the

FIG. 2. Translated protein sequence of the purified TrzN from *Arthrobacter aureescens* TC1 containing the genetically constructed His-tag region. For comparison purposes, the regions in which the *Arthrobacter* TrzN sequence differs from the published *Nocardioide*s TrzN sequence (12) are highlighted in bold print.

TABLE 1. Leaving groups displaced by TrzN

Leaving group (R)	Chemical name	Specific activity (U/mg)	Product(s)
SCH ₃	Ametryn	1.48 ± 0.11	HA, ^a CH ₃ SSCH ₃
S(O)CH ₃	Ametryn sulfoxide	1.64 ± 0.16	HA, CH ₃ SOH ^b
Cl	Atrazine	0.24 ± 0.0	HA
CN	Cyanoatrazine	0.01 ± 0.0	HA

^a HA is hydroxyatrazine.^b Methylsulfenic acid is unstable; its production was inferred from detection of methyl methanethiosulfinate.

His-tag region as purified, is shown in Fig. 2. TrzN differed from the published *Nocardioideis* sequence in 20 amino acids, 16 of which were the appended His tag and 3 of which were previously undetermined residues in the *Nocardioideis* sequence. This indicates that the *Arthrobacter* and *Nocardioideis* TrzN variants are much more similar to each other than either is to the atrazine chlorohydrolase protein, AtzA, which is typically found in gram-negative atrazine-degrading bacteria.

Range of leaving groups displaced by TrzN. AtzA has very narrow substrate specificity; it catalyzes the hydrolytic displacement of only chloride and fluoride substituents from the *s*-triazine ring (18). TrzN from *Nocardioideis* was shown to be reactive in displacing a methylmercaptan substituent (27). However, the other leaving groups tested with AtzA (fluoro-, methoxy-, cyano-, azido-, and methylsulfoxy) were not previously tested with TrzN. These leaving groups were tested here.

As previously shown for TrzN from *Nocardioideis*, ametryn, with a methylmercaptan leaving group, was an excellent substrate for the TrzN from *A. aureescens* TC1. Ametryn was hydrolyzed more rapidly than atrazine (Table 1). Interestingly, the sulfoxide analog of ametryn was an even better substrate than ametryn. Hydroxyatrazine was determined to be a product in each of these reactions. Fluoroatrazine was a substrate for TrzN as determined by spectrophotometric assay, and hydroxyatrazine was detected as a product by HPLC. An atrazine analog with a cyano substituent in place of the chlorine atom was a substrate for TrzN, albeit at rates 1 and 2 orders of magnitude lower than atrazine and ametryn, respectively (Table 1). The turnover of cyanoatrazine was confirmed by the identification of hydroxyatrazine in the enzyme reaction mixtures.

Development of a specific colorimetric assay for TrzN. The broader specificity of TrzN compared to AtzA served as the basis for the development of a colorimetric assay to distinguish TrzN- and AtzA-like atrazine chlorohydrolase activities. TrzN reaction mixtures with ametryn contained both hydroxyatrazine and methylmercaptan disulfide as products (Table 1). Methylmercaptan disulfide was likely derived from spontaneous oxidation of the primary reaction product methylmercaptan. Cell suspensions growing on ametryn had the characteristic odor of methylmercaptan. The presence of methylmercaptan was rigorously demonstrated by trapping with NBD-Cl, a reagent that reacts with methylmercaptan but not methylmercaptan disulfide. The characteristic adduct of methylthio-NBD was extracted, and its presence was demonstrated by GC-MS (*m/z* = 211). The reaction mixtures turned yellow, showing an absorbance maximum around 420 nm (Fig.

3, line b). A highly similar absorbance spectrum had previously been observed for the reaction products of other thiols with NBD-Cl (6). Subsequently, reaction mixtures were extracted and analyzed by GC-MS. We determined that characteristic peaks and mass spectra for both unreacted NBD-Cl and for an NBD adduct had precisely the molecular mass expected with the chlorine substituent replaced with an S-methyl group (Fig. 3 inset). These data confirm that the methylmercaptan disulfide product observed in ametryn-TrzN reaction mixtures is derived from the oxidative dimerization of the direct enzyme product methylmercaptan.

Ametryn sulfoxide was shown (Table 1) to be quickly turned over by TrzN with the liberation of hydroxyatrazine. The identity of the leaving group, anticipated to be methanesulfenic acid, was investigated. Previously, NBD-Cl had been used to demonstrate the formation of alkylsulfenic acids generated at the active site of enzymes (6). The addition of NBD-Cl after 30 min of reaction between TrzN and ametryn sulfoxide led to the formation of a blue-purple color. The absorbance spectrum was complex, but a major band was observed at 590 nm (Fig. 3, line c). This differed from the known product of the reaction of NBD-Cl with active site sulfenic acids. However, the present case was different in that free solution molecules of alkylsulfenic acids are known to react rapidly with each other to generate dialkylthiosulfonates (21, 22). The expected product, methyl methanethiosulfonate, was extracted into chloroform and determined by GC-MS (*m/z* = 110). Efforts to determine the identity of the chromophoric product(s) upon reaction with NBD-Cl have been unsuccessful, perhaps due to instability of the product(s). Nonetheless, the reaction is specific and diagnostic for TrzN as described below.

The formation of a colored product from a reaction product of TrzN and ametryn sulfoxide allowed us to use this reaction to test diagnostically for TrzN activity. The reaction could also be applied on freshly grown and stored whole cells of *A. aureescens* TC1 (Table 2) to determine whether TrzN activity was retained over time. Other atrazine-degrading bacteria known to contain AtzA did not show the formation of the blue color. This is consistent with previous observations that ametryn was not a substrate for AtzA (18). Incubation of ametryn sulfoxide with purified AtzA also did not yield a colored product in this assay and hydroxyatrazine was not detected, confirming that ametryn sulfoxide was not a substrate for AtzA. Other strains known to produce TrzN did produce a colored product with the assay. Thus, the present assay provides a rapid way to assess whether newly isolated atrazine-degrading bacteria contain a TrzN-like enzyme to catalyze the first reaction in the atrazine catabolic pathway.

Ring structures that serve as substrates for TrzN. Previous studies showed that AtzA catalyzes hydrolytic displacement reactions only with *s*-triazine ring substrates (18); the pyrimidines tested were not substrates. Moreover, hydrolytic chloride displacement by AtzA required the presence of at least one N-alkyl substituent in the *meta* position relative to the halogen substituent. Topp et al. (27) showed that TrzN from *Nocardioideis* required at least a *meta*-substituted N-alkyl substituent, but no pyrimidine ring substrates were tested.

In the present study, a larger range of substituent groups were tested and provided further evidence that the substrate specificity of TrzN is significantly broader than that of AtzA.

FIG. 3. Absorbance spectra of the assay products obtained with NBD-Cl added to reaction mixtures of *A. aurescens* TC1 cells and ametryn and ametryn sulfoxide, respectively. The insert shows a mass spectrum of the reaction product of ametryn and TrzN (methylmercaptan) with NBD-Cl. (a) Control with NBD-Cl and cells (dashed line); (b) NBD-Cl added to cells and ametryn (solid line); (c) NBD-Cl added to cells and ametryn sulfoxide (dotted line).

To show definitively that a tested compound was turned over, hydroxyl-triazine ring products were shown for each tested compound by HPLC analysis (Table 3). In all, 29 *s*-triazine compounds were tested; of those, 5 (simazine, propazine, terbutylazine, CEAT, and prometryn) had previously been shown to be substrates by Topp et al. (27). In the present study, 17 new compounds were shown to be substrates for TrzN (Tables 1 and 3). These data indicated that TrzN would accept considerable variation of side chain length. Moreover, 2-chloro-4,6-dimethoxy-1,3,5-triazine was a substrate for TrzN.

TABLE 2. Reactivity of different purified enzymes and atrazine-degrading bacteria with ametryn sulfoxide determined by using NBD-Cl

Component tested	Absorbance (590 nm) ^a	Disappearance of ametryn-SO ^{a,b}
Enzyme		
Purified TrzN	+	+
Purified AtzA	—	—
Purified AtzB	—	—
Purified AtzC	—	—
Bacteria		
TC1 whole cells, suspended	+	+
TC1 dried cells, hydrated	+	+
<i>Nocardioide</i> sp. strain C190	+	+
<i>Pseudomonas</i> sp. strain ADP	—	—
<i>Agrobacterium radiobacter</i> J14a	—	—
<i>Alcaligenes</i> sp. strain SG1	—	—

^a +, reactivity; —, no reactivity.

^b Disappearance of ametryn sulfoxide (ametry-SO), determined by HPLC and/or spectrophotometric assays as described in Materials and Methods.

Since a number of these additional TrzN substrates are commercial herbicides, the broader substrate specificity of TrzN is relevant to the biodegradative ability of organisms expressing TrzN in nature. Previously, *A. aurescens* TC1 was shown to grow on a broad range of *s*-triazine compounds as the sole nitrogen source (23), consistent with the findings reported here with the purified enzyme.

The best indicator of broad specificity was the demonstration of *A. aurescens* TrzN activity with 2-chloro-4,6-di-N-ethylpyrimidine. In contrast, the positional isomer, 4-chloro-2,6-di-N-ethylpyrimidine, was not a substrate. 2-Chloropyrimidines have been shown to be 60-fold more reactive than 4-chloropyrimidines in nucleophilic substitution reactions (7), suggesting that the observed difference with TrzN might be due to differential reactivity of the substrates. Previous incubations of appropriate pyrimidine substrates with AtzA and AtzC had failed to show reactivity. *s*-Triazine hydrolase from *Rhodococcus corallinus* showed low activity with chloroaminopyrimidines, but deamination, rather than dechlorination, was reported with pyrimidine substrates (11).

Conclusions. TrzN was shown to have activity with a wide range of substrates with which AtzA is not discernibly reactive. This provided the basis for the development of a colorimetric assay that can be used to discriminate the type of atrazine chlorohydrolase which is expressed by a given bacterium. The present study is also the first to purify active recombinant TrzN and thus paves the way for site-directed mutagenesis studies to probe the importance of specific amino acid residues for conferring broad substrate specificity on the TrzN-type atrazine chlorohydrolase.

TABLE 3. Compounds tested as substrates for TrzN

Structure	Substrate	Nonsubstrate		
Chlorotriazines				
	R = OCH ₃ NHCH ₂ CH ₂ OH NH(CH ₂) ₂ CH ₃ NH(CH ₂) ₃ CH ₃ NHCH ₂ CH(CH ₃) ₂ NHCH(CH ₃)CH ₂ CH ₃	C(Mo) ₂ T C(oE) ₂ T CPPT CBBT C(iB) ₂ T C(sB) ₂ T	R = NH ₂ NHCH ₃ N(CH ₃) ₂ N(CH ₃)CH ₂ CH ₃	CAAT CMMT C(MM) ₂ T C(EM) ₂ T
	R = NH ₂ NHCH ₂ CH ₃ NHCH ₂ CH ₂ OH NHC(CH ₃) ₃ NHC(CH ₃) ₂ CN	CEAT CEET C(oE)ET CE(tB)T C(2CN-1)ET		
	R = NH ₂ NHC(CH ₃) ₃	CIAT C(tB)IT		
Atrazine analogs				
	R = OCH ₃ F N ₃	(Mo)IET FIET (N ₃)IET	R = NH ₂ SH H	(NH ₂)IET SIET IE(HT)
S-methyltriazines				
	R = NHCH ₂ (CH ₃) ₂	(MS)IIT	R = NH ₂	(Ms)AAT
Pyrimidines				
	R ₁ = Cl R _{2,3} = NHCH ₂ CH ₃	CEEP	R _{1,3} = NHCH ₂ CH ₃ R _{2,3} = Cl	ECEP

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