Characterization of *S*-Triazine Herbicide Metabolism by a *Nocardioides* sp. Isolated from Agricultural Soils

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Atrazine, a herbicide widely used in corn production, is a frequently detected groundwater contaminant. Nine gram-positive bacterial strains able to use this herbicide as a sole source of nitrogen were isolated from four farms in central Canada. The strains were divided into two groups based on repetitive extragenic palindromic (rep)-PCR genomic fingerprinting with ERIC and BOXA1R primers. Based on 16S ribosomal DNA sequence analysis, both groups were identified as *Nocardioides* sp. strains. None of the isolates mineralized [*ring*-U-^{14}C]atrazine. There was no hybridization to genomic DNA from these strains using *atzABC* cloned from *Pseudomonas* sp. strain ADP or *trzA* cloned from *Rhodococcus corallinus*. *S*-Triazine degradation was studied in detail in *Nocardioides* sp. strain C190. Oxygen was not required for atrazine degradation by whole cells or cell extracts. Based on high-pressure liquid chromatography and mass spectrometric analyses of products formed from atrazine in incubations of whole cells with $H_2^{18}O$, sequential hydrolytic reactions converted atrazine to hydroxyatrazine and then to the end product *N*-ethylammelide. Isopropylamine, the putative product of the second hydrolytic reaction, supported growth as the sole carbon and nitrogen source. The triazine hydrolase from strain C190 was isolated and purified and found to have a K_m for atrazine of 25 μM and a V_{max} of 31 μmol/min/mg of protein. The subunit molecular mass of the protein was 52 kDa. Atrazine hydrolysis was not inhibited by 500 μM EDTA but was inhibited by 100 μM Mg, Cu, Co, or Zn. Whole cells and purified triazine hydrolase converted a range of chlorine or methylthio-substituted herbicides to the corresponding hydroxy derivatives. In summary, an atrazine-metabolizing *Nocardioides* sp. widely distributed in agricultural soils degrades a range of *s*-triazine herbicides by means of a novel *s*-triazine hydrolase.

The agricultural herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine; see Fig. 1 for structure) is used extensively in many parts of the world to control a variety of weeds, primarily in the production of corn. There is some evidence to suggest that atrazine may be an endocrine-disrupting chemical (10, 28). Trace levels of atrazine residues are frequently detected in surface and well water samples (19, 32, 41). Once in aquifers, it is persistent (1, 48), and thus there is considerable interest in developing agricultural management practices that minimize the potential for atrazine pollution of surface water and groundwater resources.

A variety of fungi (15, 24, 29) and bacteria (5, 7, 27, 33) which dealkylate or dechlorinate atrazine but do not mineralize the *s*-triazine ring have been isolated. Recently, there have been several reports of rapid atrazine mineralization in agricultural soils (4, 17, 44, 46), and a variety of atrazine-mineralizing bacteria, including members of the genera *Pseudomonas*, *Acinetobacter*, and *Agrobacterium*, have been isolated from soils that have come in contact with this chemical (3, 26, 27, 36, 42, 49). These bacteria commonly initiate atrazine degradation by a hydrolytic dechlorination reaction. The genes encoding an atrazine chlorohydrolase (*atzA*) and two amidohydrolytic reactions (*atzB* and *atzC*), which together convert atrazine to the ring cleavage substrate cyanuric acid, have been cloned from the *Pseudomonas* sp. strain ADP (6, 11, 38). Cyanuric acid is converted by another set of amidohydrolase enzymes to biuret and urea, which are then mineralized (9). The genes encoding

these enzymes are widespread, highly conserved, and plasmid borne in isolates that have been examined (13, 14, 23).

We are interested in agricultural management practices that influence the persistence of pesticides, and we have recently initiated a study examining the relationship of herbicide treatment history with atrazine persistence and biodegradation pathways in agricultural soils and watersheds (43). Persistence generally declines in response to herbicide use, suggesting that exposure of soil to the herbicide enhances the abundance and activity of atrazine-degrading bacteria (4, 34, 35). The objective of the work reported here was to gain a better understanding of atrazine-degrading microorganisms by characterizing the diversity, identity, and atrazine degradation mechanism of bacteria isolated from agricultural soils that have a history of exposure to atrazine.

MATERIALS AND METHODS

Sampling sites and enrichment, isolation, characterization, and maintenance of atrazine-degrading bacteria. The bacteria described in this paper were isolated from four farms located in central Canada. These consisted of a loam (site 1 at 45°22′N, 75°43′W, described in reference 44; pH 5.9; 3.0% organic matter) located near the city of Ottawa, Ontario; a sandy loam located near Winchester, Ontario (site 2 at 45°05′N, 75°40′W, described in reference 20; pH 5.7; 2.6% organic matter); a clay located near Harrow, Ontario (site 3 at 42°05′N, 82°50′W, described in reference 16; pH 5.6; 2.0% organic matter); and a loam (site 4 at 45°35′N, 73°10′W; pH 6.0; 1.4% organic matter) located near Saint Hyacinthe, Quebec. All four soils had been cropped to corn and had been treated with atrazine for weed control according to normal farming practice. Five replicate soil cores were obtained from each sample site, pooled, homogenized, and stored without drying at 4°C prior to being used for enrichment and isolation of atrazine-degrading bacteria.

Enrichment preparations consisting of a mineral salts medium containing 25 mg of atrazine/liter as the sole nitrogen and carbon source were inoculated with soil (25% wt/vol) and incubated aerobically with shaking at 30°C (45). Bacteria which formed clear zones on solidified media containing atrazine as the sole nitrogen source were purified and characterized as previously described (45).

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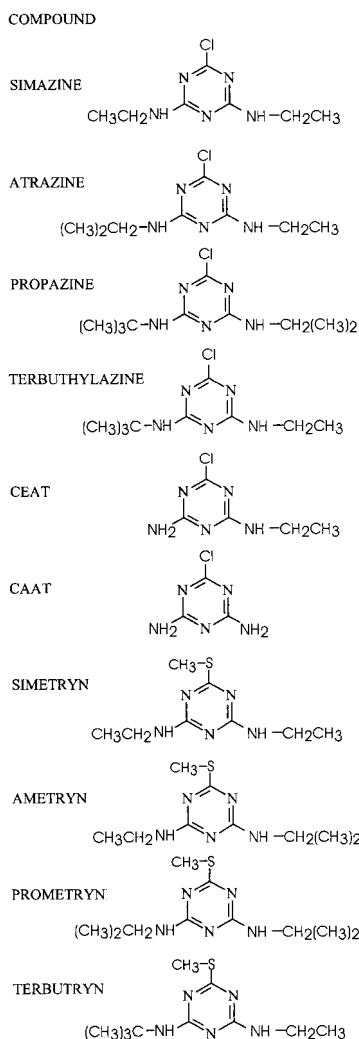
COMPOUND

SIMAZINE

CH_3CH_2NH — (triazine ring, Cl) — NH — CH_2CH_3

ATRAZINE

$(CH_3)_2CH$ — NH — (triazine ring, Cl) — NH — CH_2CH_3

PROPAZINE

$(CH_3)_3C$ — NH — (triazine ring, Cl) — NH — $CH_2(CH_3)_2$

TERBUTHYLAZINE

$(CH_3)_3C$ — NH — (triazine ring, Cl) — NH — CH_2CH_3

CEAT

NH_2 — (triazine ring, Cl) — NH — CH_2CH_3

CAAT

NH_2 — (triazine ring, Cl) — NH_2

SIMETRYN

CH_3CH_2NH — (triazine ring, CH_3—S) — NH — CH_2CH_3

AMETRYN

CH_3CH_2NH — (triazine ring, CH_3—S) — NH — $CH_2(CH_3)_2$

PROMETRYN

$(CH_3)_2CH_2NH$ — (triazine ring, CH_3—S) — NH — $CH_2(CH_3)_2$

TERBUTRYN

$(CH_3)_3C$ — NH — (triazine ring, CH_3—S) — NH — CH_2CH_3

FIG. 1. Structures of *s*-triazine compounds used in this study.

Chemicals and analytical methods. The structures of *s*-triazine compounds used in this study are presented in Fig. 1. We have adopted the nomenclature of Cook et al. (9) for the amino-substituted *s*-triazines. Analytical-grade triazine herbicides and metabolites were gifts from Novartis Crop Protection Canada Inc. (Guelph, Ontario, Canada) or were purchased from ChemService Inc. (West Chester, Pa.). Stock solutions were prepared in methanol. When added to cultures or extracts, the solvent was allowed to evaporate before the aqueous solutions were added to containers. [*ring*-U-^{14}C]atrazine (specific activity, 4.5 mCi/mmol; radioactive purity, 95%) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Water containing ^{18}O (enriched 95 to 98%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, Mass.). Parent compounds and transformation products were analyzed by reverse-phase high-pressure liquid chromatography (HPLC) on a C_{18} column using an instrument equipped with a UV detector (set at 220 nm) coupled in series with a radioactivity detector (43). The size of radioactive peaks is expressed as the integrated peak area. The solvent consisted of 70% methanol–30% 5 mM Na_2HPO_4 (pH 9.0) (solvent system 1) or 50% methanol–50% 10 mM ammonium acetate (solvent system 2). Radioactivity of samples was measured in Universol scintillation cocktail (ICN, Costa Mesa, Calif.) with a Beckman model LS5801 (Beckman, Irvine, Calif.) liquid scintillation counter using an external standard for quench correction. Mass spectra were determined by electron impact on a Finnigan-MAT 8230 mass spectrometer at an ionizing voltage of 70 eV. Metabolites were isolated and purified in preparation for mass spectral analysis by fractionating culture filtrates by HPLC, evaporating under a stream of nitrogen, and taking up the final sample in methanol. Protein was quantified by the Bradford assay (8).

Characterization of triazine herbicide degradation. Cell extracts were prepared by sonicating (four times, 2.5 min each, with 30-s rest intervals) cells resuspended in 10 mM sodium phosphate buffer (pH 7.2) and removing the undisrupted cells by centrifugation (12,000 $\times$ *g*, 12 min). In some cases, cell extracts were incubated anaerobically in serum vials sealed with grey butyl rubber stoppers by repeatedly evacuating the headspace under vacuum and backfilling to atmospheric pressure with nitrogen gas. The possible incorporation of $H_2^{18}O$ into atrazine was determined with whole cells resuspended in 100 µl of $H_2^{16}O$ or $H_2^{18}O$ and incubated aerobically overnight at 30°C with 100 µg of atrazine. Aqueous samples for chemical analysis of the parent compound and metabolites were prepared by adding methanol (50% final concentration) to cell suspensions or cell extracts and removing precipitated debris by centrifugation (14,000 $\times$ *g*, 4 min). Metabolites to be analyzed by mass spectroscopy were first isolated by HPLC fractionation using the method described below. In some cases, dechlorination of atrazine, CEAT, ametryn, and prometryn were measured spectrophotometrically at 240, 260, 250, and 252 nm, respectively. The experimentally determined values for extinction coefficients for these substrates and their dechlorinated products were as follows: atrazine, $\varepsilon_{240} = 11.2$ mM^{-1} cm^{-1}; hydroxyatrazine, $\varepsilon_{240} = 7.2$ mM^{-1} cm^{-1}; CEAT, $\varepsilon_{260} = 5.2$ mM^{-1} cm^{-1}; OEAT, $\varepsilon_{260} = <0.1$ mM^{-1} cm^{-1}; ametryn, $\varepsilon_{250} = 12$ mM^{-1} cm^{-1}; hydroxyametryn, $\varepsilon_{250} = 1.5$ mM^{-1} cm^{-1}; prometryn, $\varepsilon_{252} = 8.9$ mM^{-1} cm^{-1}; and hydroxyprometryn, $\varepsilon_{252} = 1.7$ mM^{-1} cm^{-1}. Deamination of AAAT was measured spectrophotometrically at 235 nm as described previously (31). Typical assays contained 0.5 ml of 10 mM potassium phosphate (pH 7.0) with 0.2 mM atrazine and 2 to 50 µl of cell extract and were incubated at 25°C.

DNA manipulations. Procedures for preparation of genomic DNA, blotting, and hybridizations were exactly as previously described (45). Probes for the genes *atzA*, *atzB*, *atzC*, and *trzA* were prepared from the plasmids pMD4, pATZ-2, pTD-2, and pSWP1, respectively (6, 11, 38, 40). A 0.9-kb probe for *trzA* was prepared by cutting plasmid pSWP1 with *Nco*I and *Kpn*I. Purified plasmids were digested with restriction enzymes (pMD4, *Apa*I and *Pst*I; pATZ-2, *Bgl*II and *Eco*RI; pTD-2, *Cla*I and *Hinc*II; pSWP1, *Nco*I and *Kpn*I) by standard procedures (39). After being electrophoretically separated in 1% low-melting-point multipurpose agarose (Roche Molecular Biochemicals, Laval, Quebec, Canada), a 0.6-kb internal fragment of the *atzA* gene, a 1.2-kb internal fragment of the *atzB* gene, a 0.75-kb internal fragment of the *atzC* gene, and a 0.9-kb internal fragment of *atzA* were extracted using an agarose gel DNA extraction kit (Roche Molecular Biochemicals). The purified fragments were labeled with digoxigenin (DIG) by random priming using the DIG high primer DNA labeling and detection starter kit II (Roche Molecular Biochemicals) as specified by the manufacturer.

PCR genomic fingerprinting was done with ERIC (21) and BOXA1R (47) primers as previously described (45). PCR amplification, cloning, sequencing, and analysis of the entire 16S rRNA gene were done exactly as previously described (45). The following bacteria were included in the phylogenetic analysis: *Aeromicrobium erythreum*, accession number AF005021; *Aeromicrobium fastidiosum*, Z78209; *Actinoplanes utahensis*, X80823; *Nocardioides jensenii*, AF005006; *Nocardioides plantarum*, AF005008; *Nocardioides simplex*, AF005009; *Rhodococcus globerulus*, X81931; *Nocardioides* sp. strain C157, AF253509; *Nocardioides* sp. strain C190, AF253510; and *Streptomyces lividans*, X86354.

Purification of the triazine hydrolase. Cells from 4 liters of *Nocardioides* culture which had been grown to stationary phase were pelleted by centrifugation (4,000 $\times$ *g*, 10 min, 4°C). The 4-g pellet was resuspended with 8 ml of ice-cold 10 mM potassium phosphate (KP$_i$) (pH 7.0) (buffer A). This cell suspension was passed three times through a chilled French pressure cell (15,000 lb/in^2), and whole cells and debris were removed by centrifugation (12,000 $\times$ *g*, 10 min, 4°C). The supernatant was subjected to ultracentrifugation (105,000 $\times$ *g*, 1 h, 4°C), and the supernatant from this treatment (crude soluble fraction) was removed and used as a source of triazine hydrolase for further purification. The crude soluble fraction was pumped (3 ml/min) onto a TSK-DEAE anion-exchange column (30.0 by 2.5 cm) (Waters/Millipore, Milford, Mass.) that had been equilibrated with buffer A. After the column was washed with 200 ml of buffer A, a linear gradient of 0 to 1.0 M NaCl (pH 7.0) in buffer A was run (5 ml/min) to elute bound material from the column. Fractions containing triazine hydrolase activity were pooled and used for further purification. The pooled DEAE fractions containing hydrolase activity were brought to 1 M $(NH_4)_2SO_4$ by the addition of solid $(NH_4)_2SO_4$ and were pumped at 3 ml/min onto a TSK-phenyl column (2.15 by 15 cm) (HP-Genenchem, South San Francisco, Calif.) that had been equilibrated with buffer A containing 1 M $(NH_4)_2SO_4$. After the column was washed with 200 ml of equilibration buffer, a linear gradient of 1.0 to 0 M $(NH_4)_2SO_4$ in buffer A was run at 5 ml/min to elute bound material from the column. Peak active fractions were pooled and diluted 10-fold with buffer A. The pooled diluted phenyl fractions were again subjected to ion-exchange chromatography on a TSK-DEAE column as described above. Peak active fractions were stored at 4°C.

Size exclusion chromatography of crude extracts and purified hydrolase fractions was carried out with a Biosep SEC3000 column (21.5 by 600 mm) (Phenomenex) equilibrated and run with buffer A containing 0.2 M NaCl at 6 ml/min. Pooled DEAE and phenyl fractions were concentrated using Centricon-30 microconcentrators (Amicon, Beverly, Mass.), and 0.1- to 0.2-ml aliquots of this concentrate were injected onto the column. Fractions containing hydrolase activity were pooled. For estimation of the native molecular size of the hydrolase, the column was calibrated with prepared protein standards (Bio-Rad).

Protein determinations and SDS-PAGE. Protein concentrations in partially purified and purified enzyme preparations were determined by the spectrophotometric method of Kalb and Bernlohr (22). Sodium dodecyl sulfate-polyacryl-
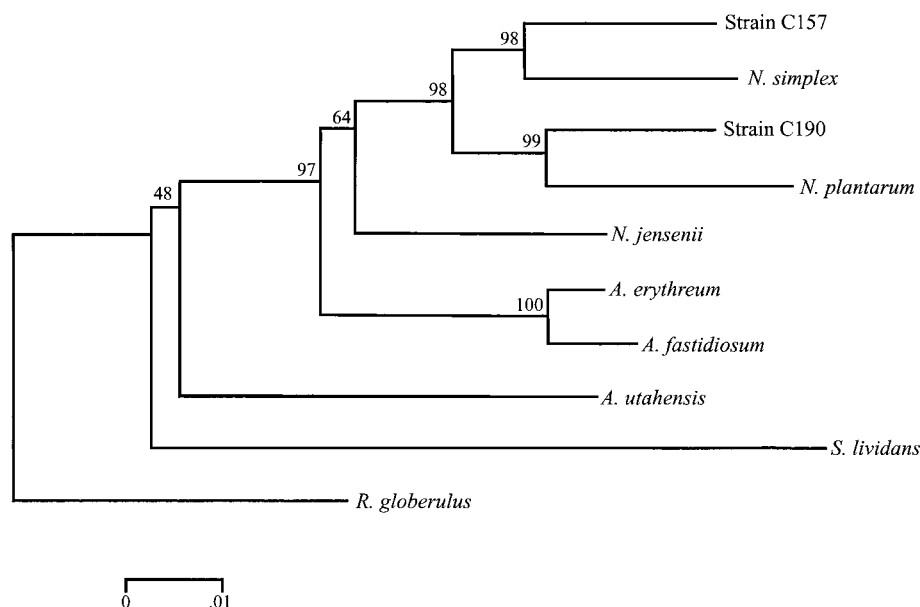
FIG. 2. Phylogenetic tree based on the 16S ribosomal DNA sequence data showing the relationships of strains C157 and C190 with the most closely related bacteria identified in the GenBank database. Included in the analysis are *Actinoplanes utahensis*, *Aeromicroblum fastidiosum*, *N. simplex*, *N. plantarum*, *Nocardioides albus*, *N. jensenii*, *S. lividans*, and *R. globerulus* as an outlier. The bar indicates 0.01 substitutions per nucleotide position.

amide gel electrophoresis (SDS-PAGE; 4 to 12% acrylamide gradient gel) of proteins was performed by the method of Laemmli (25), and fragments were sized using the Bio-Rad mid-range protein standard kit.

RESULTS

Identity of atrazine-degrading bacteria. Nine gram-positive atrazine-degrading bacteria which formed clear zones on the atrazine agar medium were isolated and characterized; four isolates were obtained from site 1, three were from site 2, and one each was from sites 3 and 4. Colonies were uniform in appearance, and after 3 weeks of growth on atrazine-mineral salts (AMS) medium, they formed 2-mm-diameter colonies with the following properties: buff, dull, opaque appearance, circular form, convex elevation, entire margin, and viscid consistency without mycelial growth. Cellular morphology was consistent; all isolates were short, nonmotile, nonpleiotrophic gram-positive rods when recovered from AMS agar.

Rep-PCR fingerprinting with the ERIC and the BOXA1R primers revealed one group of two siblings and a second group of seven siblings. Strains C157 and C158, isolated from site 1, had identical ERIC and the BOXA1R fingerprints. The other seven isolates, strains C194 and C196 from site 1, strains C189 and C190 from site 2, strain C183 from site 3, and strain C188 from site 4, likewise had identical ERIC and the BOXA1R fingerprints. The 16S rRNA gene from a representative isolate of each of the two PCR fingerprint types, strains C157 and C190, was sequenced to obtain information on their identity. When aligned with the sequences available in the GenBank database, strains C157 and C190 were identified as *Nocardioides* sp. (Fig. 2). Strain C190 was most similar to *Nocardioides plantarum*, and strain C157 most closely related to *Nocardioides simplex*.

Pathway and mechanism of atrazine degradation, and s-triazine substrate specificity. Atrazine was incompletely degraded by these bacteria; none of them converted [*ring*-U-^{14}C] atrazine to carbon dioxide (data not shown). Strain C190 was chosen for detailed study.

Dense (adjusted to an A_{600} of 2) cell suspensions degraded 20 mg of atrazine/liter in an overnight incubation (data not shown). Atrazine degradation by cell suspensions or by cell extracts did not require oxygen (data not shown). HPLC analyses of resting cell suspensions of strain C190 incubated with 10 mg of [*ring*-U-^{14}C]atrazine/liter revealed a metabolite (I) which coeluted with hydroxyatrazine (retention times, 4.7 min [solvent system 1] and 5.9 min [solvent system 2]) and then an end product metabolite (II) which was more hydrophilic and eluted at the solvent front in both solvent systems (Fig. 3). Deethyl- and deisopropylatrazine were not detected in culture filtrates. Metabolites I and II were purified by HPLC fractionation and were subjected to solid-probe mass spectrometry. Metabolite I had a molecular ion (M$^+$, base peak) at m/z 197 and major peaks at m/z 182, 155, 140, 127, 112, 97, 84, 71, and
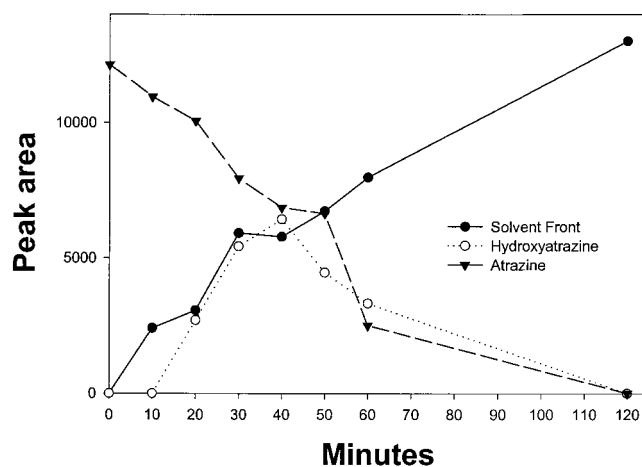

FIG. 3. Disposition of radioactivity during metabolism of [*ring*-U-^{14}C]atrazine by whole cells of strain C190. Radioactivity is expressed as the area integrated under peaks.

58 and had a mass spectrum corresponding exactly to that of an analytical-grade hydroxyatrazine standard (data not shown). Metabolite II had a mass spectrum identical to that of an analytical standard of *N*-ethylammelide (molecular formula, $C_5H_8N_4O_2$), with a molecular ion at m/z 156 (M^+, base peak) and major fragments at m/z 141 (loss of CH_3), 128 (loss of CH), 112 (loss of O) and 98 (loss of N) (Fig. 4). The source of the hydroxyl groups in the *N*-ethylammelide was determined by incubating a resting cell suspension of strain C190 for 24 h with atrazine in $H_2^{18}O$. The mass spectrum of the recovered metabolite was similar to that of *N*-ethylammelide but with major peaks larger by 4 or 2 mass units (Fig. 4), consistent with a molecular formula of $C_5H_8N_4^{18}O_2$. The molecular ion was at m/z 160 (M^+, base peak), with major peaks at m/z 145 (loss of CH_3), 132 (loss of CH), 114 (loss of ^{18}O), and 100 (loss of N). The mass spectrum of analytical *N*-ethylammelide incubated for 24 h at 30°C in $H_2^{18}O$ was identical to that of the analytical standard, indicating that exchange had not taken place (data not shown). These results indicate that both hydroxyl groups in the *N*-ethylammelide originate from water, as illustrated in Fig. 5.

Strain C190's ability to grow on components of atrazine in solidified mineral salts medium was consistent with the proposed pathway. Two grams of isopropylamine/liter but not 2 g of ethylamine/liter supported growth as the sole carbon and nitrogen source. Two grams of cyanuric acid/liter did not support growth as the sole nitrogen source (data not shown).

Genomic DNAs of all strains were tested in a dot blot assay for the ability to hybridize to DIG-labeled sequences from the *atzA*, *atzB*, and *atzC* genes of *Pseudomonas* strain ADP and *trzA* from *Rhodococcus corallinus*. None of the strains hybridized with any of the probes (data not shown). There is a 405-bp region of *atzA* that is highly conserved in all atrazine-hydrolyzing gram-negative bacteria and in *Clavibacter michiganensis* ATZ1 and that is detectable by PCR amplification with specific primers (13). These primers failed to amplify genomic DNA of strain C190, whereas they did for *Pseudomonas* strain ADP (data not shown).

Strain C190 degraded a range of chloro- and methylthio-substituted *s*-triazines (Fig. 1; Table 1). All substrates were degraded by anaerobic cell suspensions (data not shown). The methylthio-substituted herbicides were degraded by both whole cells and cell extract more rapidly than were the corresponding chlorinated analogs. Metabolites recovered from cultures were more hydrophilic than the parent compounds and had mass spectral characteristics consistent with the loss of the methylthio group (47 mass units) and the acquisition of a hydroxy group (17 mass units; total net loss, 30 mass units) (Table 2). In the case of ametryn and terbutryn, the metabolites had characteristics identical to those metabolites produced from the corresponding chloro-substituted herbicides. Taken together, the results indicate that the methylthio group of these herbicides is removed hydrolytically, yielding the corresponding hydroxytriazine products.

Isolation and purification of triazine hydrolase enzyme. Purification of the triazine hydrolase was accomplished by chromatography of the crude soluble fraction on a DEAE anion-exchange column, followed by chromatography of active fractions on TSK-phenyl and DEAE columns (Table 3). The final preparation had a specific activity approximately 10 times that of the starting material and yielded a single band of approximately 52,000 Da when subjected to SDS-PAGE (Fig. 6).

Enzyme characterization. The native molecular size of the triazine hydrolase was estimated by size exclusion chromatography to be approximately 75,000 Da. From these results, it is unclear whether the enzyme is composed of a single subunit
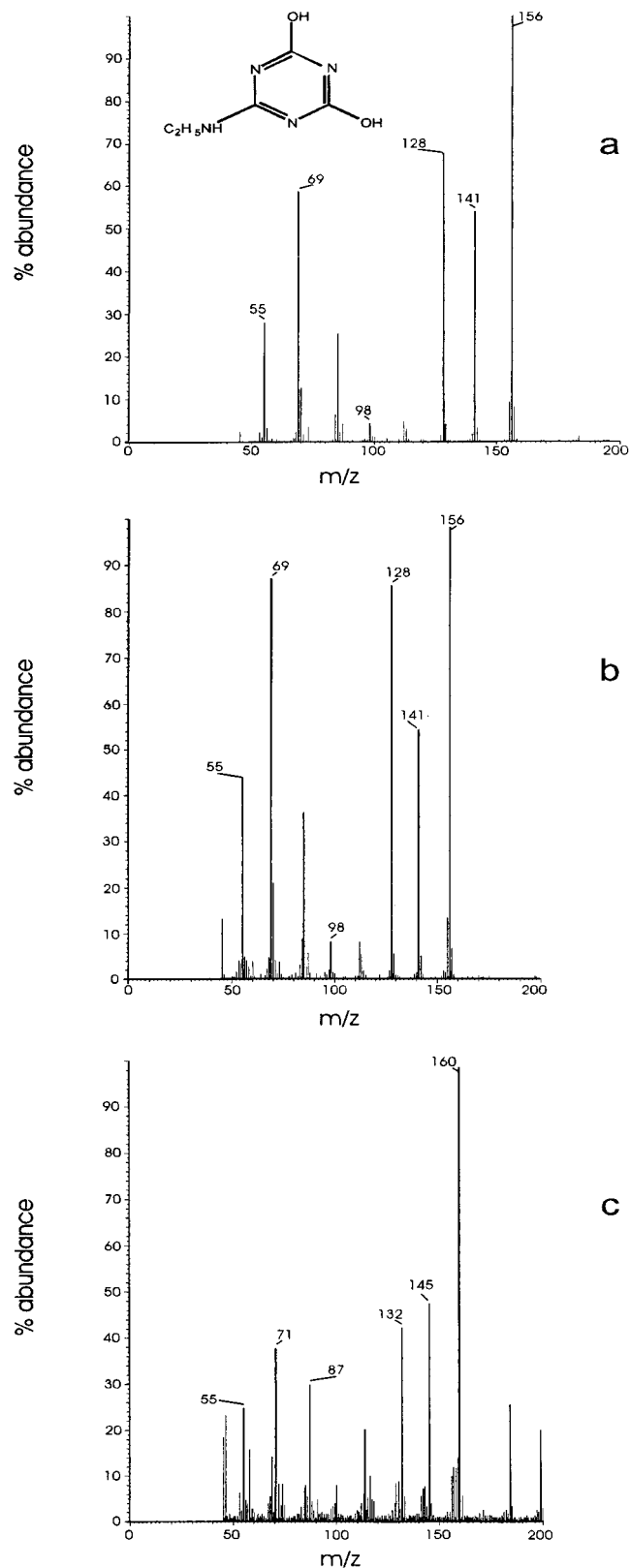

FIG. 4. Mass spectra of an *N*-ethylammelide analytical standard (a) and of the end product of atrazine metabolism recovered from culture filtrates of *Nocardioides* strain C190 incubated in $H_2^{16}O$ (b) or in $H_2^{18}O$ (c).
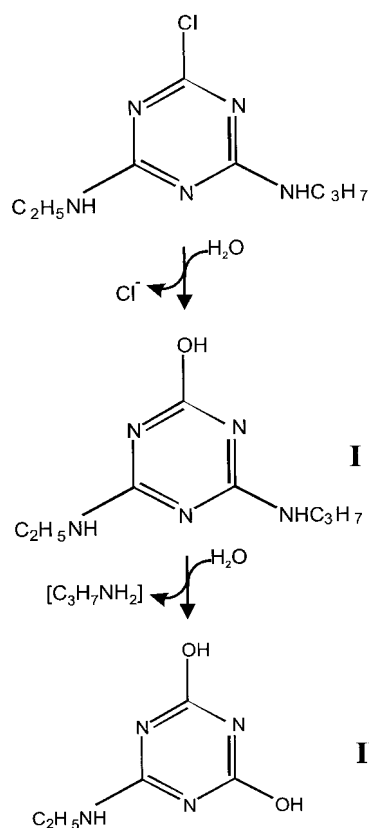
FIG. 5. Proposed pathway of atrazine metabolism by *Nocardioides* sp. strain C190. Metabolite I is hydroxyatrazine, and metabolite II is *N*-ethylammelide.

with the total size of 52,000 Da or is a dimer with a total size of 104,000 Da.

In assays designed to test the effect of different metal salts or divalent metal chelators, MnSO$_4$ and EDTA showed no effect on enzyme activity at 100 μM and 500 μM, respectively. However, assays containing 100 μM MgSO$_4$, CuSO$_4$, CoSO$_4$, or ZnSO$_4$ showed inhibition of hydrolase activity by these metal salts (approximately 20, 50, 70, and 80% inhibition, respectively).

In order to estimate the substrate specificity of the triazine hydrolase, Michaelis-Menten constants were estimated from

TABLE 1. Relative rates of triazine hydrolysis by whole cells, cell extracts, and purified triazine hydrolase

Substrate	Relative rate of triazine hydrolysis[a]		Enzyme value	
	Whole cells	Cell extract	K_m (μM)	V_{max} (μmol/min/mg of protein)
Atrazine	100	100	25	31
Simazine	95	119	ND[b]	ND
Propazine	17	5	ND	ND
Terbuthylazine	50	60	ND	ND
Ametryn	193	194	17	18
Simetryn	167	198	ND	ND
Prometryn	212	75	310	98
Terbutryn	123	32	ND	ND
CEAT	ND	ND	131	0.35

[a] Given as percentages of the atrazine rate.
[b] ND, not done.

TABLE 2. Characteristics of methylthio-*s*-triazine substrates and of metabolites accumulated after overnight incubation of suspensions of *Nocardioides* sp. strain C190[a]

Substrate	Characteristics of substrate		Characteristics of product	
	HPLC retention time (min)	*m/z* (% intensity)	HPLC retention time (min)	*m/z* (% intensity)
Atrazine	7.2	215 (M$^+$, 75), 200 (100), 173 (20), 132, 18, 71 (24), 58 (58)	4.6	197 (M$^+$, 100), 182 (90), 155 (45), 127 (36), 112 (80), 69 (52), 58 (92)
Terbuthylazine	7.9	229 (40), 214 (M$^+$, 100), 173 (50), 132 (30), 71 (42)	5.4	211 (80), 196 (M$^+$, 100), 169 (12), 155 (80), 126 (72), 112 (30), 69 (30), 58 (42)
Simetryn	8.1	213 (M$^+$, 100), 198 (24), 170 (50), 155 (42), 96 (35), 71 (53), 68 (84)	4.0	183 (M$^+$, 10), 149 (22), 111 (15), 85 (45), 70 (55), 55 (100)
Ametryn	8.7	227 (M$^+$, 100), 212 (64), 185 (35), 170 (40), 99 (25), 68 (60), 58 (64)	4.6	197 (M$^+$, 100), 182 (90), 155 (35), 127 (25), 112 (62), 69 (70), 57 (96)
Prometryn	9.3	241 (M$^+$, 100), 226 (60), 199 (30), 184 (72), 106 (25), 58 (64)	4.8	211 (M$^+$, 100), 196 (94), 169 (42), 154 (58), 112 (65), 69 (45), 58 (88)
Terbutryn	15.0	241 (M$^+$, 70), 226 (90), 185 (100), 170 (55), 106 (32), 68 (60)	5.8	211 (M$^+$, 62), 196 (100), 155 (72), 126 (64), 69 (28)

[a] The initial substrate concentration was 10 mg/liter.

TABLE 3. Purification of triazine hydrolase from
Nocardioides sp. strain C190

Purification step	Total protein (mg)	Total activity (IU)	Sp act (IU/mg of protein)	Recovery (%)	Purification (fold)
Crude soluble	5.94	14.2	2.39	100	
TSK-DEAE	5.6	8.9	1.59	66	
TSK-phenyl	0.95	4.4	4.60	31	1.9
TSK-DEAE	0.08	1.8	22.5	12	9.4

least-squares regression of Lineweaver-Burk plots using the structurally related *s*-triazines atrazine, ametryn, prometryn, and CEAT (Table 1). The enzyme also had activity toward the *s*-triazines terbuthylazine and propazine, but we were unable to determine kinetic constants using these compounds because of their low aqueous solubilities. The enzyme displayed no detectable dechlorination activity in assays containing the triazine CAAT and no detectable deamination activity toward CAAT or AAAT. We did not test whether these compounds were competitive inhibitors of the dechlorination of atrazine.

DISCUSSION

In this study, nine atrazine-degrading gram-positive isolates were obtained from four agricultural soils, clustered into two groups by rep-PCR fingerprinting, and identified as *Nocardioides* sp. strains on the basis of 16S ribosomal DNA sequencing. The fact that these atrazine-degrading *Nocardioides* sp. strains were isolated from four independent farms in central Canada suggests that these organisms are geographically widespread. *Nocardioides* species have previously been shown to degrade a variety of toxic organic pollutants, including 2,4,6-trinitrophenol (picric acid), 2,4,5-trichlorophenoxyacetic acid, and the organophosphorus insecticide coumaphos (18, 30, 37).

The *Nocardioides* sp. strains converted atrazine through hydroxyatrazine to the end product *N*-ethylammelide by means of an atrazine chlorohydrolase and a hydroxyatrazine *N*-isopropylaminehydrolase (Fig. 5). This proposed pathway is supported by several lines of evidence: ring-labeled atrazine was not mineralized, atrazine degradation did not require oxygen, the herbicide was converted to hydroxyatrazine and then to *N*-ethylammelide by whole cells, the two hydroxyl groups in the *N*-ethylammelide end product originated from water, and isopropylamine but not ethylamine or cyanuric acid was metabolized. Previously reported atrazine-degrading gram-positive bacteria N-dealkylate atrazine by means of a P450-like monooxygenase (33). The absence of detection of deethyl- or deisopropylatrazine in supernatants of cultures that have degraded atrazine further suggests that *Nocardioides* does not transform atrazine by this mechanism. *R. corallinus* NRRLB-15444R produces a triazine chlorohydrolase, but atrazine is not transformed by this enzyme (31). Remarkably, the *Nocardioides* atrazine-degradation pathway appears to be identical to that recently reported for *Clavibacter michiganensis* ATZ1, part of an atrazine-mineralizing consortium isolated from an agricultural soil (12). Strain ATZ1 was identified on the basis of fatty acid methyl ester analysis, and it has a similarity index of 0.584 with *C. michiganensis* when compared with the MIDI database (2). Although the pathways of atrazine degradation are identical, the genes encoding the enzymes are not; *atzABC* genes were undetectable by hybridization in *Nocardioides*, whereas *C. michiganensis* possesses sequences homologous to these genes (13). *atzABC* genes have been found in all atrazine chlorohydrolase-expressing bacteria examined to date, including

Pseudomonas, *Alcaligenes*, *Ralstonia*, *Agrobacterium*, and *Pseudaminobacter* (12, 13, 42, 45). Although the hydrolytic mechanism of *s*-triazine transformation is conserved in *Nocardioides*, our results indicate that there is hitherto-undetected diversity in the genes encoding the enzyme.

In a related study, two of the soils which yielded the *Nocardioides* sp. (sites 1 and 4) also yielded a *Pseudaminobacter* sp. which mineralized atrazine, the "upper pathway" consisting of hydrolases encoded by *atzABC* (45). It was noted in that report that the alkylamine-degrading *Pseudaminobacter* sp. would have the selective advantage of being able to utilize atrazine as a carbon source upon the acquisition of *atzABC*. The results reported in this study indicate that these very same soils contain at least two distinct populations which can use atrazine as both a carbon and a nitrogen source. It is noteworthy that although the genes encoding the atrazine-degrading enzymes are not shared by these bacteria, the metabolic strategy of hydrolytically cleaving growth-supporting alkylamine groups from the *s*-triazine ring is conserved.

The ability of strain C190 to degrade methylthio-substituted *s*-triazine herbicides makes it distinct from *Pseudomonas* strain ADP, which cannot (26). Recently, a range of genera including *Clavibacter*, *Alcaligenes*, and *Agrobacterium* expressing homologs of AtzA were found to vary significantly in their *s*-triazine substrate specificity, in spite of sharing a nearly
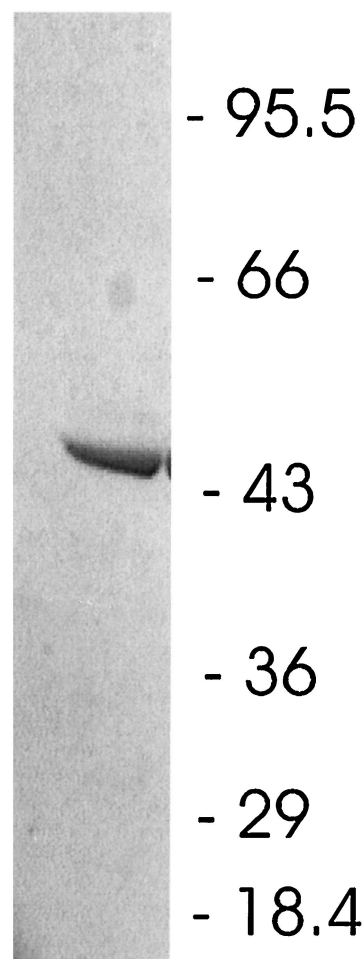

FIG. 6. SDS-PAGE gel of purified triazine hydrolase. The numbers correspond to the molecular masses (in kilodaltons) of protein standards.

identical 500-bp conserved region in the chlorohydrolase genes (J. L. Seffernick, M. J. Sadowsky, and L. P. Wackett, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999, abstr. K-62, p. 412, 1999). Analysis of the chlorohydrolase-encoding gene from strain C190 will help elucidate sequences specifying substrate specificity and hydrolytic activity. We are currently attempting to locate and clone this gene.

In summary, our results indicate that a *Nocardioides* sp. isolated from atrazine-treated agricultural soils degrades a variety of *s*-triazine herbicides by means of a novel chlorohydrolase and that there is hitherto-undetected diversity in atrazine chlorohydrolase-encoding genes.

ACKNOWLEDGMENTS

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