JOURNAL OF BACTERIOLOGY, Aug. 1996, p. 4894–4900 Vol. 178, No. 16
0021-9193/96/$04.00+0

Atrazine Chlorohydrolase from *Pseudomonas* sp. Strain ADP: Gene Sequence, Enzyme Purification, and Protein Characterization†

MERVYN L. DE SOUZA,[1,2] MICHAEL J. SADOWSKY,[2,3,4] AND LAWRENCE P. WACKETT[1,2,4]*

Department of Biochemistry and Biological Processes Technology Institute,[1] Department of Soil, Water, and Climate,[3] Department of Microbiology,[4] and Center for Biodegradation Research and Informatics,[2] University of Minnesota, St. Paul, Minnesota 55108

Received 26 April 1996/Accepted 30 May 1996

***Pseudomonas* sp. strain ADP metabolizes atrazine to carbon dioxide and ammonia via the intermediate hydroxyatrazine. The genetic potential to produce hydroxyatrazine was previously attributed to a 1.9-kb *Ava*I DNA fragment from strain ADP (M. L. de Souza, L. P. Wackett, K. L. Boundy-Mills, R. T. Mandelbaum, and M. J. Sadowsky, Appl. Environ. Microbiol. 61:3373–3378, 1995). In this study, sequence analysis of the 1.9-kb *Ava*I fragment indicated that a single open reading frame, *atzA*, encoded an activity transforming atrazine to hydroxyatrazine. The open reading frame for the chlorohydrolase was determined by sequencing to be 1,419 nucleotides and encodes a 473-amino-acid protein with a predicted subunit molecular weight of 52,421. The deduced amino acid sequence matched the first 10 amino acids determined by protein microsequencing. The protein AtzA was purified to homogeneity by ammonium sulfate precipitation and anion-exchange chromatography. The subunit and holoenzyme molecular weights were 60,000 and 245,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration chromatography, respectively. The purified enzyme in $H_2{}^{18}O$ yielded [^{18}O]hydroxyatrazine, indicating that AtzA is a chlorohydrolase and not an oxygenase. The most related protein sequence in GenBank was that of TrzA, 41% identity, from *Rhodococcus corallinus* NRRL B-15444R. TrzA catalyzes the deamination of melamine and the dechlorination of deethylatrazine and desisopropylatrazine but is not active with atrazine. AtzA catalyzes the dechlorination of atrazine, simazine, and desethylatrazine but is not active with melamine, terbutylazine, or desethyldesisopropylatrazine. Our results indicate that AtzA is a novel atrazine-dechlorinating enzyme with fairly restricted substrate specificity and contributes to the microbial hydrolysis of atrazine to hydroxyatrazine in soils and groundwater.**

The *s*-triazine ring is found as a constituent of herbicides, dyes, and polymers. The *s*-triazine herbicides include simazine, terbutylazine, and atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine)]. The latter is the most environmentally prevalent, being used for the control of broadleaf and grassy weeds in major crops like corn, sorghum, and sugarcane. Atrazine is relatively persistent in soils (34). Atrazine occasionally exceeds the U.S. Environmental Protection Agency maximum contaminant level of 3 ppb in groundwater and surface water (1, 5, 6, 14, 21, 22, 23, 30, 31).

The metabolism of *s*-triazine compounds by pure bacterial cultures has been studied (3, 4, 8–10, 15, 17, 20, 25–27, 32, 41, 42), but most isolates failed to metabolize atrazine (9, 15). In general, less-substituted *s*-triazine ring compounds are more readily metabolized than their heavily substituted counterparts. As a result, information about the microbial genetics and enzymology of *s*-triazine compounds has largely been obtained with compounds other than atrazine. For example, ammeline (2-hydroxy-4,6-diamino-*s*-triazine), which is not alkylated on the ring-substituted amino groups, is metabolized by *Pseudomonas* sp. strain NRRL B 12227 (12, 13). Its metabolism proceeds to cyanuric acid via two hydrolytic deamination reactions that are encoded by the genes *trzB* and *trzC*. Diamino-*s*-triazines with one alkylamino group, such as desethylsimazine and desethylatrazine are metabolized by *Rhodococcus corallinus* NRRL B-15444R (8) via a hydrolytic enzyme that catalyzes both dechlorination and deamination reactions (28). The gene encoding this hydrolase, *trzA*, was recently cloned and sequenced (36). However, both *Pseudomonas* sp. strain NRRLB 12227 and *R. corallinus* NRRL B-15444R were incapable of metabolizing atrazine. Recently, bacteria that metabolize atrazine have been isolated (29, 32, 35, 36), but little information is available about the relevant metabolites, genes, and enzymes.

We previously reported the isolation of a pure bacterial culture, identified as *Pseudomonas* sp. strain ADP, which degraded relatively high concentrations of atrazine (>1,000 ppm) under growth and nongrowth conditions (25). *Pseudomonas* sp. strain ADP uses atrazine as the sole source of nitrogen for growth and liberates the ring carbon atoms as carbon dioxide. Recently, we reported the cloning, characterization, and expression of a DNA fragment from strain ADP that confers atrazine dechlorination ability on *Escherichia coli* DH5α (11). The data indicate that hydroxyatrazine was the first intermediate in the metabolism of atrazine by *Pseudomonas* sp. strain ADP.

The present study describes the sequence of the gene encoding the atrazine dechlorination activity, designated *atzA*, and the purification of the corresponding enzyme (AtzA). Atrazine chlorohydrolase was characterized with respect to physical and catalytic properties. This is the first description of a purified bacterial enzyme capable of catalyzing atrazine dechlorination.

* Corresponding author. Mailing address: Department of Biochemistry, University of Minnesota, 140 Gortner Laboratories, 1479 Gortner Ave., St. Paul, MN 55108. Phone: (612) 625-3785. Fax: (612) 625-5780. Electronic mail address: wackett@biosci.cbs.umn.edu.

† Manuscript number 22,472 in the University of Minnesota Agricultural Experiment Station series.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Plasmid pMD4 (11), containing a 1.9-kb *Ava*I fragment in pACYC184 (7), was used in all studies. *E. coli* DH5α(pMD4) was used for the purification of atrazine chlorohydrolase. The culture was grown in Luria-Bertani LB medium (33), supplemented with 25 μg of chloramphenicol per ml, at 37°C with shaking.

DNA sequencing. Plasmid DNA was isolated as described previously (33). The nucleotide sequence of the approximately 1.9-kb *Ava*I DNA fragment, cloned in pMD4, was determined from both strands by using a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer Corp., Norwalk, Conn.) and an ABI model 373A DNA sequencer (Applied Biosystems, Foster City, Calif.). Nucleotide sequence was determined initially by subcloning and subsequently by using primers designed with sequence information obtained from subcloned DNA fragments. The GCG sequence analysis software package (Genetics Computer Group, Inc., Madison, Wis.) was used for all DNA and protein sequence comparisons. DNA and protein sequences were compared with entries in Genbank, EMBL, PIR, and SwissProt sequence databases.

Analytical methods. (i) Plate assays. Atrazine or hydroxyatrazine was incorporated in solid LB (33) or minimal medium (25) at a final concentration of 500 μg/ml to produce an opaque suspension of small particles in the clear agar. The degradation of atrazine or hydroxyatrazine by wild-type and recombinant bacteria was indicated by a zone of clearing around atrazine-metabolizing colonies (11).

(ii) HPLC analysis. High-performance liquid chromatography (HPLC) analysis was performed by using a Hewlett-Packard HP 1090 Liquid Chromatograph system equipped with a photodiode array detector and interfaced to an HP 79994A Chemstation. Atrazine and its metabolites were resolved by using an analytical C_{18} reverse-phase NovaPak HPLC column (150 by 3.9 mm; Waters) 4-μm spherical packing; and an acetonitrile (ACN) gradient, in water, at a flow rate of 1.0 ml min^{-1}. Linear gradients were used as follows: 0 to 6 min, 10 to 25% ACN; 6 to 21 min, 25 to 65% ACN; 21 to 23 min, 65 to 100% ACN; and 23 to 25 min, 100% ACN. Spectral data of the column eluent were acquired between 200 and 400 nm (12-nm bandwidth per channel), with a sampling frequency of 640 ms. Spectra were referenced against a signal at 550 nm and compared with those obtained by using authentic samples of atrazine and hydroxyatrazine.

(iii) MS analysis. $H_2{}^{18}O$ (95%) and $H_2{}^{16}O$ were purchased from Sigma Chemical Company (St. Louis, Mo.). For the labeling experiment, 50 μl of authentic atrazine or hydroxyatrazine at a concentration of 100 μg/ml in 25 mM MOPS buffer (pH 6.9) was added to each vial in two sets of glass vials. The samples were dried-down under vacuum and crimp sealed. The headspaces in the vials were repeatedly evacuated and flushed and finally filled with argon. The contents of each vial was resuspended in 50 μl of either $H_2{}^{18}O$ or $H_2{}^{16}O$ by using a gas-tight Hamilton syringe and allowed to equilibrate at room temperature for 60 min. The experiment was initiated by adding purified atrazine chlorohydrolase (AtzA) to each vial. Reaction vials were shaken for 30 s and incubated at room temperature for 5 h, and the reaction mixture was dried under vacuum. The reaction mixture was resuspended in 25 μl of methanol. Direct-insertion mass spectrometry (Ms) was performed with a Kratos mass spectrometer (Kratos, Ramsey, N.J.) in a glycerol matrix operated in the fast atom bombardment mode with xenon. Control experiments were performed in $H_2{}^{18}O$ to test for spontaneous exchange of ^{18}O from water into the hydroxyl group of hydroxyatrazine in the absence and presence of purified AtzA.

Chemicals. Authentic samples of atrazine (2-chloro-4-ethylamino-6-isopropyl amino-1,3,5-*s*-triazine), desethylatrazine (2-chloro-4-amino-6-isopropylamino-*s*-triazine), deisopropylatrazine (2-chloro-4-ethylamino-6-amino-*s*-triazine), hydroxyatrazine (2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine), desethylhydroxyatrazine (2-hydroxy-4-amino-6-isopropylamino-*s*-triazine), desisopropylhydroxyatrazine (2-hydroxy-4-amino-6-isopropylamino-*s*-triazine), desethyldesisopropylatrazine (2-chloro-4,6-diamino-*s*-triazine), simazine (2-chloro-4,6-diethylamino-*s*-triazine), terbutylazine (2-chloro-4-ethylamino-6-terbutylamino-*s*-triazine, ametryn (2-ethylamino-4-isopropylamino-6-methylthio-*s*-triazine), prometryn [2,4-bis(isopropylamino)-6-(methylthio)-*s*-triazine], a sulfonyl urea herbicide {1-(4-methoxy-6-methyl-triasin-2-yl)-3-[2-(3,3,3-trifluoropropyl)-phenylsulfonyl]-urea}, triasulfuron [*N*-(6-methoxy-4-methyl-1,3,5-triazin-2-yl-aminocarbonyl)-2-(2-chloroethoxy)-benzenesulfonamide], cyromazine (*N*-cyclopropyl-1,3,5-triazine-2,4,6-triamine), and melamine (2,4,6-triamino-*s*-triazine) were obtained from Ciba-Geigy Corp., Greensboro, N.C. All other chemicals used were reagent grade or better.

Protein purification. *E. coli* DH5α(pMD4) was grown overnight at 37°C in LB medium containing 25 μg of chloramphenicol per ml. The culture was centrifuged at 10,000 × *g* for 10 min at 4°C and washed in 0.85% NaCl, and the cell pellet was resuspended in 25 mM MOPS buffer (pH 6.9) containing phenylmethylsulfonyl fluoride (100 μg/ml). Cells were broken by passage three times through a Amicon French pressure cell at 20,000 lb/in^2 at 4°C. Cell extract was obtained by centrifugation at 10,000 × *g* for 15 min. The supernatant was clarified by centrifugation at 18,000 × *g* for 90 min. The crude cell extract was chilled to 4°C, and solid NH_4SO_4 was added, with stirring, to attain 20% saturation. The solution was stirred for 30 min at 4°C and centrifuged at 12,000 × *g* for 20 min. The precipitated material was resuspended in 25 mM MOPS buffer (pH 6.9) and dialyzed overnight at 4°C against 25 mM MOPS buffer, pH 6.9.

```
   1  CTCGGGTAAC TTCTTGAGCG CGGCCACAGC AGCCTTGATC ATGAAGGCGA
  51  GCATGGTGAC CTTGACGCCG CTCTTTTCGT TCTCTTTGTT GAACTGCACG
 101  CGAAAGGCTT CCAGGTCGGT GATGTCCGCG TCGTCGTGGT TGGTGACGTG
 151  CGGGATGACC ACCCAGTTGC GGTGCAGGTT TTTCGATGGC ATAATATCTG
                                        atzA →
 201  CGTTGCGACG TGTAACACAC TATTGGAGAC ATATCATGCA AACGCTCAGC
 251  ATCCAGCACG GTACCCTCGT CACGATGGAT CAGTACCGCA GAGTCCTTGG
 301  GGATAGCTGG GTTCACGTGC AGGATGGACG GATCGTCGCG CTCGGAGTGC
 351  ACGCCGAGTC GGTGCCTCCG CCAGCGGATC GGGTGATCGA TGCACGCGGC
 401  AAGGTCGTGT TACCCGGTTT CATCAATGCC CACACCCATG TGAACCAGAT
 451  CCTCCTGCGC GGAGGGCCCT CGCACGGACG TCAATTCTAT GACTGGCTGT
 501  TCAACGTTGT GTATCCGGGA CAAAAGGCGA TGAGACCGGA GGACGTAGCG
 551  GTGGCGGTGA GGTTGTATTG TGCGGAAGCT GTGCGCAGCG GGATTACGAC
 601  GATCAACGAA AACGCCGATT CGGCCATCTA CCCAGGCAAC ATCGAGGCCG
 651  CGATGGCGGT CTATGGTGAG GTGGGTGTGA GGGTCGTCTA CGCCCGCATG
 701  TTCTTTGATC GGATGGACGG GCGCATTCAA GGGTATGTGG ACGCCTTGAA
 751  GGCTCGCTCT CCCCAAGTCG AACTGTGCTC GATCATGGAG GAAACGGCTG
 801  TGGCCAAAGA TCGGATCACA GCCCTGTCAG ATCAGTATCA TGGCACGGCA
 851  GGAGGTCGTA TATCAGTTTG GCCCGCTCCT GCCACTACCA CGGCGGTGAC
 901  AGTTGAAGGA ATGCGATGGG CACAAGCCTT CGCCCGTGAT CGGGCGGTAA
 951  TGTGGACGCT TCACATGGCG GAGAGCGATC ATGATGAGCG GATTCATGGG
1001  ATGAGTCCCG CCGAGTACAT GGAGTGTTAC GGACTCTTGG ATGAGCGTCT
1051  GCAGGTCGCG CATTGCGTGT ACTTTGACCG GAAGGATGTT CGGCTGCTGC
1101  ACCGCCACAA TGTGAAGGTC GCGTCGCAGG TTGTGAGCAA TGCCTACCTC
1151  GGCTCAGGGG TGGCCCCCGT GCCAGAGATG GTGGAGCGCG GCATGGCCGT
1201  GGGCATTGGA ACAGATAACG GGAATAGTAA TGACTCCGCA AACATGATCG
1251  GAGACATGAA GTTTATGGCC CATATTCACC GCGCGGTGCA TCGGGATGCG
1301  GACGTGCTGA CCCCAGAGAA GATTCTTGAA ATGGCGACGA TCGATGGGGC
1351  GCGTTCGTTG GGAATGGACC ACGAGATTGG TTCCATCGAA ACCGGCAAGC
1401  GCGCGGACCT TATCCTGCTT GACCTGCGTC ACCTCAGACG ACTCTCACAT
1451  CATTTGGCGG CCACGATCGT GTTTCAGGCT TACGGCAATG AGGTGGACAC
1501  TGTCCTGATT GACGGAAACG TTGTGATGGA GAACCGCCGC TTGAGCTTTC
1551  TTCCCCCTGA ACGTGAGTTG GCGTTCCTTG AGGAAGCGCA GAGCCGCGCC
1601  ACAGCTATTT TGCAGCGGGC GAACATGGTG GCTAACCCAG CTTGGCGCAG
1651  CCTCTAGGAA ATGACGCCGT TGCTGCATCC GCCGCCCCTT GAGGAAATCG
1701  CTGCCATCTT GGCGCGGCTC GGATTGGGGG GCGGACATGA CCTTGATGGA
1751  TACAGAATTG CCATGAATGC GGCACTTCCG TCCTTCGCTC GTGTGGAATC
1801  GTTGGTAGGT GAGGGTCGAC TGCGGGCGCC AGCTTCCCGA AGAGGTGAAA
1851  GGCCCGAG
```

FIG. 1. Nucleotide sequence of *atzA*. The complete nucleotide sequence of the approximately 1.9-kb *Ava*I DNA fragment, cloned in pMD4, was determined on both strands by the primer walking method and PCR. The *atzA* ORF is indicated by the arrow, and a potential *Pseudomonas* ribosome binding site is underlined. The start and stop codons are underlined twice.

The dialyzed protein fraction was loaded onto a Bio-Scale Q20 Anion Exchange column (15 by 118 mm) (Bio-Rad Laboratories, Hercules, Calif.). The column was washed with 25 mM MOPS buffer (pH 6.9), and the protein was eluted using a 400-ml linear gradient of 0.0 to 0.5 M KCl at a flow rate of 2 ml/min. Protein eluting from the column was monitored at 280 nm by using a Pharmacia U.V. Protein Detector (Pharmacia, Uppsala, Sweden). Pooled fractions containing the major peak were dialyzed overnight against 25 mM MOPS buffer, pH 6.9. The dialyzed material was concentrated by ultrafiltration, assayed for activity in transforming atrazine to hydroxyatrazine by HPLC, and analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (24).

Protein characterization. The molecular weights of protein subunits were determined by SDS-PAGE by comparison to known standard proteins, using a MiniProtean II gel apparatus (Bio-Rad). The size of the holoenzyme was determined by gel filtration chromatography on a Superose 6 HR column (1.0 by 30.0 cm), using an FPLC System (Pharmacia). The column was equilibrated in 25 mM MOPS buffer (pH 6.9) containing 0.1 M NaCl. Proteins with known molecular weights (apoferritin, β-amylase, alcohol dehydrogenase, and bovine serum albumin) were used as chromatography standards to calibrate the column. Isoelectric point determinations were performed with a Pharmacia Phast-gel System and Pharmacia IEF 3-9 media. A Pharmacia broad-range pI calibration kit was used for standards. The absorption maximum and extinction coefficient were determined with a Beckman DU 640 spectrophotometer (Fullerton, Calif.). The protein concentration was determined by quantitative amino acid analysis. The extinction coefficient is expressed per molar subunit.

Amino acid analysis. The amino acid composition and N-terminal amino acid sequence of purified AtzA were determined with a Beckman 6300 Amino Acid Analyzer at the Microchemical Facility, Human Genetics Institute, University of Minnesota, Minneapolis.

Metal analysis. Purified AtzA (7.72 mg) was dissolved in 500 μl of reagent-grade HCl by vortexing under vacuum for 5 min. The solution was sealed in a glass vial and subjected to acid hydrolysis at 110°C for 24 h. The vial was cooled to room temperature, opened, and diluted to 5 ml. The metal content of the diluted solution was determined by inductively coupled plasma emission spectroscopy at the Soil Analytical Laboratory of the University of Minnesota, St. Paul.

Enzyme kinetics. Purified AtzA, 6.5 μg, was added to different concentrations of atrazine in 500-μl reaction volumes, and the reactions were allowed to proceed at room temperature for 2, 5, 7, and 10 min. Reactions were stopped by the addition of 500 μl of ACN and rapidly cooled to −80°C. Thawed samples were

```
AtzA  1 MQTLSIQHGTLVTMDQYRRVLGDSWVHVQDGRIVALGVHAESVPP..... 45
TrzA    MTRIAITGGRVLTMDPERRVLEPGTVVVEDQFIAQVGSPTTSTSAAPKSS 50

     46 .PADRVIDARGKVVLPGFINAHTHVNQILLRGGPSHGRQFYDWLFNVVYP 94
     51 TPPGWQCSPASSTPTPTSHKSSSGVVHP.MTATSSNGCTTCS..IPASLP 97

     95 GQKAMR.PEDVAVAVRLYCAEAVRSGITTINENADSAIYPGNIEAAMAVY 143
     98 TQTTTSESEHCCTAPKPFVLASPLSSTTRTSDPTTSP....APGPPGSPF 143

    144 GEVGVRVVYARMFFDRMDGRIQGYVDALKARSPQVELCSIMEETAVAKDR 193
    144 TDAGIRAIYARMYFDAPRAELEELVATIHAKAPGAVRMDESASTDHVLAD 193

    194 ITALSDQYHGTAGGRISVWPAPATTTAVTVEGMRWAQAFARDRAVMWTLH 243
    194 LDQLITRHDRTADGRIRVWPAPAIPFMVSEKGMKAAQEIAASRTDGWTMH 243

    244 MAESDHDERIHGMSPAEYMECYGLLDERLQVAHCVYFDRKDVRLLHRHNV 293
    244 VSEDPIEARVHSMNAPEYLHHLGCLDDRLLAAHCVHIDSRDIRLFRQHDV 293

    294 KVASQVVSNAYLGSGVAPVPEMVERGMAVGIGTDNGNSNDSANMIGDMKF 343
    294 KISTQPVSNSYLAAGIAPVPEMLAHGVTVGIGTDDANCNDSVNLISDMKV 343

    344 MAHIHRAVHRDADVLTPEKILEMATIDGARSLGMDHEIGSIETGKRADLI 393
    344 LALIHRAAHRDASIITPEKIIEMATIDGARCIGMADQIGSLEAGKRADII 393

    394 LLDLRHLRRL.SHHLAATIVFQAYGNEVDTVLIDGNVVMENRRLSFLP.P 441
    394 TLDLRHAQTTPAHDLAATIVFQAYGNEVNDVLVNGSVVMRDRVLSFLPTP 443

    442 ERELAFLEEAQSRATAILQRANMVANPAWRSL.. 473
    444 QEEKALYDDASERSAAMLARAGLTGTRTWQTLGS 477
```

FIG. 2. Amino acid sequence alignment of AtzA (top lines) and TrzA from *R. corallinus* NRRL B-15444R (bottom lines) (28). Identical amino acids are indicated by vertical lines. Gaps have been introduced to optimize the alignment.

centrifuged at 14,000 rpm for 10 min in a Beckman Instruments Microfuge E, the supernatants were filtered through a 0.2-μm-pore-size filter, and placed in crimp-seal HPLC vials. HPLC analysis was used to calculate initial rates of atrazine disappearance and hydroxyatrazine formation. Enzyme kinetic parameters were determined by double-reciprocal plots. The K_m obtained was nearly equivalent to the aqueous solubility of atrazine. Therefore, kinetic parameters were estimated from substrate concentrations below K_m.

Nucleotide sequence accession number. The nucleotide sequence of *atzA* and the protein sequence of AtzA are accessible in the GenBank Nucleotide Sequence Database under accession number U55933.

RESULTS

DNA and protein sequences of the *atzA* gene. The nucleotide sequence of the approximately 1.9-kb *Ava*I DNA fragment in pMD4 determined by sequencing both strands is shown in Fig. 1. Several possible open reading frames (ORFs) were observed. However, the results of codon usage and preference analysis and previous Tn*5* data (11), suggested that one large ORF, beginning at base number 236, comprised the atrazine chlorohydrolase gene. This gene, *atzA*, consisted of 1,419 nucleotides that encoded a polypeptide of 473 amino acids with a calculated molecular weight of 52,421 and a pI of 6.6. A canonical *Pseudomonas* ribosome binding site, beginning with GGAGA, is located 11 bp upstream from the proposed translational start codon. A putative stop codon is located at position 1655.

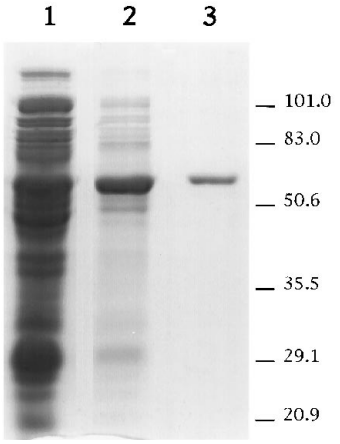

FIG. 3. Denaturing PAGE with protein samples obtained during various stages of purification of AtzA. Lanes: 1, crude cell extract; 2, 0 to 20% ammonium sulfate pellet; 3, concentrated peak active fractions from Q20 anion-exchange chromatography. Molecular mass markers were phosphorylase *b* (101,000), bovine serum albumin (83,000), ovalbumin (50,600), carbonic anhydrase (35,500), soybean trypsin inhibitor (29,100), and lysozyme (20,900). The numbers to the right of the gel are kilodaltons.

The amino acid sequence derived from the translation of *atzA* is shown in Fig. 2. N-terminal sequence analysis of purified AtzA protein indicated that the first 10 amino acids detected (MQTLSIQHGT) were identical to those predicted by translation of the *atzA* ORF described above.

Homology of AtzA to other proteins. The amino acid sequence of atrazine chlorohydrolase, derived from translation of *atzA*, was compared with those of other proteins in the SwissProt and PIR databases and translated genes in the GenBank and EMBL databases. AtzA had the highest sequence identity, 41% at the amino acid level, with translated TrzA from *R. corallinus* NRRL B-1544R and low, nonsignificant amino acid sequence identity (less than 22%) with other entries in the database. The alignment of AtzA and TrzA is shown in Fig. 2. Sequence alignment indicated a higher degree of amino acid conservation toward the C termini of the proteins.

Purification of AtzA. The atrazine chlorohydrolase was reproducibly purified from a cell extract of *E. coli*(pMD4) by precipitation with $(NH_4)_2SO_4$ added to 20% saturation. The pellet formed by using 20% $(NH_4)_2SO_4$ was redissolved in MOPS buffer (pH 6.9) and dialyzed against the same buffer. The dialyzed 20% fraction was further purified by anion-exchange chromatography on a Bio-Scale Q20 column. AtzA eluted as a major peak with a 0 to 0.5 M KCl gradient. Peak

TABLE 1. Purification of atrazine chlorohydrolase (AtzA) from *E. coli* DH5α(pMD4)

Purification step	Total amt (mg) of protein	Total activity (U)[a]	Sp act (U/mg of protein)	Recovery (%)	Fold purification
Crude soluble	1,318.5	275.0	0.208	100.0	1.0
Ammonium sulfate pellet (20% saturation)	278.2	187.2	0.673	68.0	3.2
Bio-Scale Q20 column	17.3	72.0	4.5	38.5	22.0

[a] 1 unit of enzyme activity is defined as the conversion of 1 μmol of atrazine to hydroxyatrazine per min at room temperature.

TABLE 2. Molecular properties of purified AtzA

Property	Value for atrazine chlorohydrolase
Molecular weight	
Holoenzyme	
Gel filtration	245,000
Subunit	
SDS-PAGE	60,000
Calculated[a]	52,421
Subunit structure	$(\alpha)_4$ or $(\alpha)_5$
Metal content	<0.1[b]
pI	
Observed	5.3
Calculated[a]	6.6
Absorption maximum	280 nm
Extinction coefficient[c]	63,652

[a] Values predicted from the translated nucleic acid sequence of *atzA*, using the GCG sequence analysis software package.
[b] <0.1 mol of metal per subunit for the following metals: Al, B, Ca, Cd, Co, Cr, Cu, Mg, Mn, Ni, Pb, and Zn.
[c] Per molar subunit.

fractions were concentrated and found to yield a single major band with a molecular weight of approximately 60,000 when subjected to SDS-PAGE (Fig. 3). The final preparation had a specific activity approximately 22 times that of the starting material (Table 1).

Enzyme characterization. The molecular weight of the native protein was estimated by gel filtration chromatography on a Superose 6HR column to be approximately 245,000 (results not shown). Denaturing PAGE indicated that the subunit molecular weight was 60,000. The subunit molecular weight deduced from the translated DNA sequence was 52,421 and because this is likely to be a precise determination (Table 2), the native enzyme is a homotetramer or homopentamer. No metals were detected in stoichiometric amounts with native enzyme isolated from *E. coli*(pMD4). The isoelectric point of the protein was found to be 5.25 (Table 2). The yield and fold purification obtained for AtzA suggest that it accounts for 4.6% of the soluble protein in *E. coli* DH5α(pMD4) (Table 1).

Purified AtzA catalyzed the dechlorination of atrazine to hydroxyatrazine via a hydrolytic reaction. Atrazine, incubated for 5 h with purified AtzA in $H_2{}^{18}O$ or $H_2{}^{16}O$ under an argon atmosphere, quantitatively yielded [^{18}O]- or [^{16}O]hydroxyatrazine respectively, as demonstrated by fast atom bombardment MS (Fig. 4). The major peaks at *m/z* 200 (199 + 1) and 198 (197 + 1) indicated the incorporation of ^{18}O and ^{16}O from $H_2{}^{18}O$ and $H_2{}^{16}O$, respectively, during atrazine dechlorination. Authentic hydroxyatrazine incubated with purified AtzA in $H_2{}^{18}O$ or $H_2{}^{16}O$ was not transformed. In addition, authentic hydroxyatrazine solubilized in $H_2{}^{18}O$ without added AtzA did not reveal any spontaneous exchange of ^{18}O into the hydroxyl groups during a 5-h incubation period. The minor peak at *m/z* 198 in Fig. 4B was caused by the $H_2{}^{16}O$ contamination from the purified AtzA preparation. Authentic hydroxyatrazine yielded a mass spectrum with the expected hydroxyatrazine peak at *m/z* 198 (197 + 1).

AtzA was also examined for its ability to hydrolyze various triazine compounds in vitro by HPLC analysis. Only atrazine,

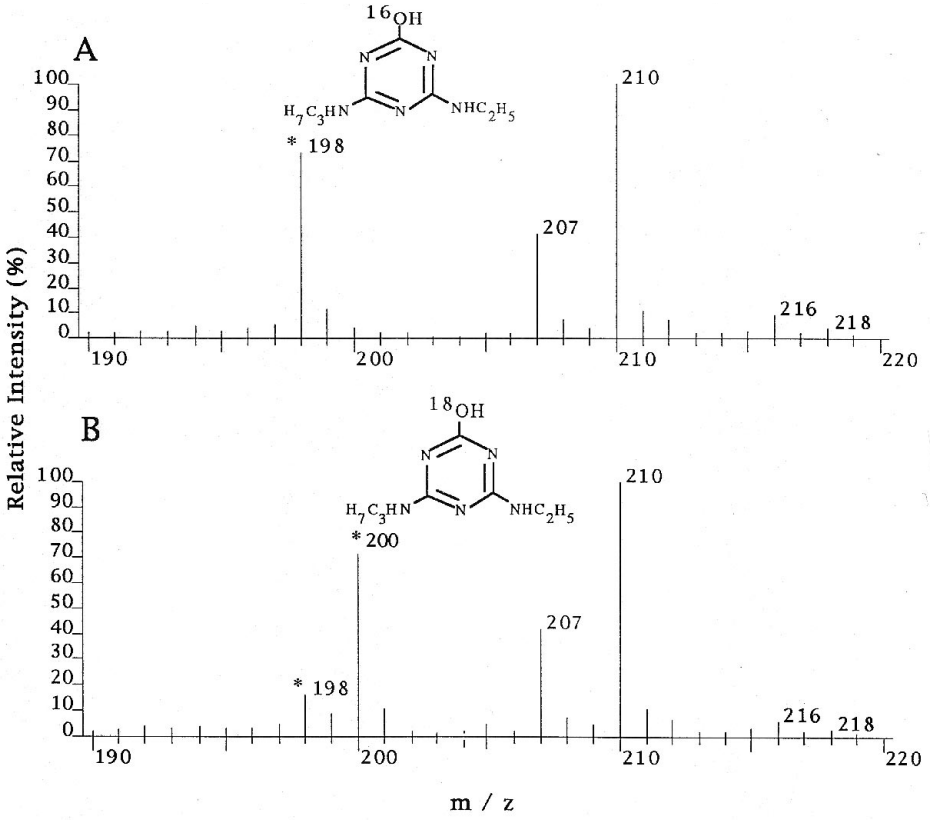

FIG. 4. Fast atom bombardment mass spectra of hydroxyatrazine formed from atrazine incubated with atrazine chlorohydrolase, AtzA, in $H_2{}^{16}O$ (A) and $H_2{}^{18}O$ (B). The peak at *m/z* 207 represents two molecules of glycerol and sodium, and the peak at *m/z* 210 is from MOPS buffer (209.3 + 1). The peaks marked with an asterisk at *m/z* 198 and 200 represent [^{16}O]- or [^{18}O]hydroxyatrazine formed by incubation of atrazine with AtzA. The peaks at *m/z* 216 and 218 represent residual atrazine.

TABLE 3. Predicted and protein-derived amino acid compositions of AtzA from *Pseudomonas* sp. strain ADP expressed in *E. coli*

Residue	No. of amino acid residues	
	Predicted from DNA sequence	Determined by protein hydrolysis
A = Ala	53	50
B = Asx	47[a]	46[a]
C = Cys	4	4
F = Phe	11	15
G = Gly	35	46
H = His	20	18
I = Ile	25	26
K = Lys	9	16
L = Leu	36	39
M = Met	21	18
P = Pro	18	22
R = Arg	39	35
S = Ser	23	23
T = Thr	21	26
V = Val	48	46
W = Trp	6	ND[b]
Y = Tyr	14	17
Z = Glx	43[c]	47[c]

[a] Combination of Asp and Asn.
[b] ND, not determined by method.
[c] Combination of Glu and Gln.

deethylatrazine, deisopropylatrazine, and simazine were hydrolyzed by purified AtzA. All of these substrates contain a chlorine atom and one or more alkylamino (C_2 or C_3) side chains. Desethyldesisopropylatrazine, melamine, terbutylazine, ametryn, prometryn, cyromazine, triasulfuron, and a sulfonylurea herbicide were not substrates for AtzA. Kinetic experiments revealed that melamine did not inhibit the dechlorination of atrazine by AtzA (data not shown).

Enzyme kinetics. The K_m and V_{max} of AtzA for atrazine were 149 μM and 2.6 μmol of hydroxyatrazine per min per mg of protein, respectively. The k_{cat}, based on a holoenzyme molecular weight of 245,000, was 11 s^{-1}.

DISCUSSION

In this report, we describe the purification and characterization of a unique atrazine chlorohydrolase (AtzA), encoded by a 1.9-kb *Ava*I genomic DNA fragment from *Pseudomonas* sp. strain ADP. Nucleotide sequence analysis indicated that *atzA* is 1,419 nucleotides and encodes a polypeptide of 473 amino acids. While several ORFs were found in the DNA sequence, one large ORF beginning at base number 236 was established by several criteria as the start site for atrazine chlorohydrolase. (i) *E. coli* transformed with the 1.9-kb fragment cloned in pMD4 gained the ability to hydrolyze atrazine to hydroxyatrazine. (ii) The dechlorination activity was abolished by transposon Tn*5* insertions specifically within the indicated ORF on the 1.9-kb *Ava*I fragment (11). (iii) The N-terminal residues of AtzA determined by amino acid analysis were identical with the predicted 10 N-terminal residues of the translated gene *atzA*. (iv) There was a good correlation between the predicted and observed amino acid compositions of atrazine chlorohydrolase (Table 3). (v) There was reasonable agreement between the predicted and observed molecular masses of the subunit and holoenzyme of AtzA.

The DNA sequence upstream of the putative translation start site for *atzA* did not show similarities to previously identified *E. coli* promoter sequences by the method of Staden (39). However, a potential *Pseudomonas* ribosome binding site was found 11 bp upstream of the ATG codon (37). AtzA had the highest amino acid sequence identity (41%) with TrzA, a dechlorinating enzyme from *R. corallinus* NRRL B-15444R. This is not surprising, since both enzymes catalyze hydrolytic reactions with triazine substrates.

AtzA was purified to homogeneity, in two steps, by precipitation with 20% saturated $(NH_4)_2SO_4$ and anion-exchange chromatography. The fact that AtzA is precipitated from cell-free supernatants at such a low concentration of $(NH_4)_2SO_4$ is surprising and fortuitous and will facilitate the large-scale production of AtzA for potential applications in remediating atrazine-contaminated soils or waters.

AtzA and TrzA have a significant degree of amino acid similarity and catalyze hydrolytic reactions with structurally similar substrates (Table 4), but there are major differences between these two hydrolases. The K_m of AtzA for atrazine is 149 μM. The related triazine hydrolase, TrzA, showed a preference for monoalkylated triazines over dialkylated triazines (28). This was evident from K_m values of 82 and 61 μM for desethylsimazine (desisopropylatrazine) and desethylatrazine, respectively, compared with K_i values of 385 and 746 μM for atrazine and simazine, respectively. In fact, the primary activity of TrzA is reported to be the deamination of compounds like melamine (28). Melamine was not deaminated by AtzA and did not inhibit the dechlorination of atrazine (data not shown). A number of pyrimidine compounds were spectrophotometrically evaluated as potential substrates for deamination and dechlorination by AtzA, but no activity was detected (data not shown). In total, it appears that substrate hydrolysis by AtzA requires an *s*-triazine ring structure, a chlorine substituent, and an alkylamino substituent.

The dechlorination of atrazine to yield hydroxyatrazine has been observed in plants and animals. Hydroxyatrazine was one of the 19 urinary metabolites observed during the metabolism of atrazine in rats (2). Turkey tissues (18) and chicken liver homogenates (16, 22) have both been reported to metabolize atrazine to hydroxyatrazine. Three pathways have been proposed for atrazine detoxification by corn plants, one is hypothesized to be a nonenzymatic reaction with benzoxazinone that undergoes spontaneous decomposition to hydroxyatrazine (38). Firm evidence for the precise biochemical mechanism

TABLE 4. Properties of AtzA and TrzA[a]

Enzyme	Substrates	Products	Reactions catalyzed	Mol wt		No. of subunits
				Holoenzyme	Subunit	
s-Triazine hydrolase (TrzA)	Melamine and deethylsimazine	Ammeline and *N*-ethylammeline	Deamination and dechlorination	200,000	54,000	4
Atrazine chlorohydrolase (AtzA)	Atrazine	Hydroxyatrazine	Dechlorination	240,000	52,421	4 or 5

[a] AtzA from *Pseudomonas* sp. strain ADP and TrzA from *R. corallinus* NRRL B-1544R. Data for TrzA from reference 28.

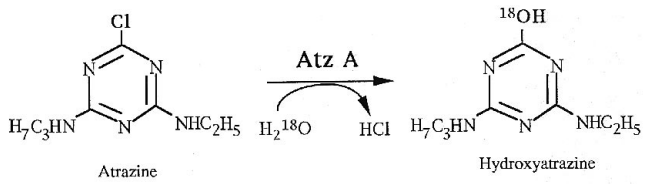

FIG. 5. The first enzymatic step in atrazine degradation by *Pseudomonas* sp. strain ADP, encoded by *atzA* located on pMD4 generates, via a hydrolytic mechanism, hydroxyatrazine which is subsequently metabolized to carbon dioxide and ammonia (25).

underlying hydroxyatrazine formation in these nonbacterial systems must still be found, so the present study may lead the way in elucidating these details.

Pseudomonas sp. strain ADP was enriched for and isolated from a mixed culture that could dechlorinate atrazine to hydroxyatrazine under both aerobic and oxygen-limited conditions and yield [^{18}O]hydroxyatrazine in $H_2{}^{18}O$ (27). This result suggested a hydrolytic dechlorination mechanism, but data were not previously obtained with pure enzyme or even pure bacterial cultures. It is of interest that the dechlorination of pentachlorophenol to tetrachlorohydroquinone was originally thought to proceed via a hydrolytic mechanism (40) and later was shown to be catalyzed by a monooxygenase (41). Any ambiguity about atrazine dechlorination was resolved by experiments using $H_2{}^{18}O$ and an oxygen-deficient atmosphere. Authentic hydroxyatrazine solubilized in $H_2{}^{18}O$ did not reveal any spontaneous exchange of ^{18}O into the hydroxyl group. However, atrazine incubated with purified AtzA in $H_2{}^{18}O$ or $H_2{}^{16}O$ in an argon environment quantitatively yielded [^{18}O]- or [^{16}O]hydroxyatrazine, respectively, confirming the hydrolytic mechanism of the reaction catalyzed by AtzA. Moreover, hydroxyatrazine incubated with purified AtzA in $H_2{}^{18}O$ did not yield any detectable [^{18}O]hydroxyatrazine. This result rules out the possibility that the enzyme catalyzed exchange after an initial monooxygenation reaction that used traces of oxygen that remained in sealed reaction vessels. These results are supported by earlier evidence of hydroxyatrazine accumulation in cultures of *E. coli*(pMD4) (11). Taken together, the data conclusively demonstrated that AtzA catalyzes the hydrolytic dechlorination of atrazine (Fig. 5).

Hydroxyatrazine is thought to be nonherbicidal and nontoxic (19) and does not leach from soil as readily as atrazine (15). Thus, hydrolytic dechlorination is an ideal mechanism for metabolizing atrazine with the goal of environmental restoration. Furthermore, studies with enzymes like AtzA that catalyze hydrolytic dehalogenation reactions further our understanding of the molecular basis of environmental organochlorine metabolism. In summary, we present the first report of the sequence of a gene encoding atrazine chlorohydrolase (AtzA), the purification of AtzA from the atrazine-degrading bacterium *Pseudomonas* sp. strain ADP, and the first definitive evidence for the enzymatic mechanism underlying atrazine dechlorination.

ACKNOWLEDGMENTS

This work was supported in part by a grant from Ciba-Geigy Corporation and by grant 94-34339-1122 from the U.S. Department of Agriculture-BARD program as grant number US-2364-93 from BARD, the United States-Israel Binational Agricultural Research and Development Fund.

We thank Janis McFarland and Steven Dumford of Ciba-Geigy Corporation for providing *s*-triazine compounds; Keith Miller, Bill Koskinen, and Tom Krick for experimental assistance; and Lisa Newman and Lynda Ellis for helpful discussions.

REFERENCES

1. **Baker, B. D., and P. R. Richards.** 1992. Herbicide concentrations in Ohio's drinking water supplies: a quantitative exposure assessment, p. 84–97. *In* J. H. Lehr (ed.), Rational readings on environmental concerns. Van-Nostrand Reinhold, New York.
2. **Bakke, J. E., J. D. Larson, and C. E. Price.** 1972. Metabolism of atrazine and 2-hydroxyatrazine by the rat. J. Agric. Food Chem. **20:**602–607.
3. **Behki, R., E. Topp, W. Dick, and P. Germon.** 1993. Metabolism of the herbicide atrazine by *Rhodococcus* strains. Appl. Environ. Microbiol. **59:** 1955–1959.
4. **Behki, R. M., and S. U. Kahn.** 1986. Degradation of atrazine by *Pseudomonas*: n-dealkylation and dehalogenation of atrazine and its metabolites. J. Agric. Food Chem. **34:**746–749.
5. **Belluck, D. A., S. L. Benjamin, and T. Dawson.** 1991. Groundwater contamination by atrazine and its metabolites: risk assessment, policy, and legal implications, p. 254–273. *In* L. Somasundaram and J. R. Coats (ed.), Pesticide transformation products: fate and significance in the environment. American Chemical Society, Washington, D.C.
6. **Buser, H. R.** 1990. Atrazine and other *s*-triazine herbicides in lakes and rain in Switzerland. Environ. Sci. Technol. **24:**1049–1058.
7. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
8. **Cook, A., and R. Hutter.** 1984. Deethylsimazine: bacterial dechlorination, deamination and complete degradation. J. Agric. Food Chem. **32:**581–585.
9. **Cook, A. M.** 1987. Biodegradation of *s*-triazine xenobiotics. FEMS Microbiol. Rev. **46:**93–116.
10. **Cook, A. M., and R. Hutter.** 1981. *s*-Triazines as nitrogen sources for bacteria. J. Agric. Food Chem. **29:**1135–1143.
11. **de Souza, M. L., L. P. Wackett, K. L. Boundy-Mills, R. T. Mandelbaum, and M. J. Sadowsky.** 1995. Cloning, characterization, and expression of a gene region from *Pseudomonas* sp. strain ADP involved in the dechlorination of atrazine. Appl. Environ. Microbiol. **61:**3373–3378.
12. **Eaton, R. W., and J. S. Karns.** 1991. Cloning and analysis of *s*-triazine catabolic genes from *Pseudomonas* sp. strain NRRLB-12227. J. Bacteriol. **173:**1215–1222.
13. **Eaton, R. W., and J. S. Karns.** 1991. Cloning and comparison of the DNA encoding ammelide aminohydrolase and cyanuric acid aminohydrolase from three *s*-triazine-degrading bacterial strains. J. Bacteriol. **173:**1363–1366.
14. **Eisler, R.** 1989. Atrazine hazards to fish, wildlife, and invertebrates. A synoptic review. Contaminant Hazard Review report 18. Fish and Wildlife Service, U.S. Department of the Interior, Washington, D.C.
15. **Erickson, E. L., and K. H. Lee.** 1989. Degradation of atrazine and related *s*-triazines. Crit. Rev. Environ. Control **19:**1–13.
16. **Foster, T. S., S. U. Khan, and M. H. Akhtar.** 1979. Metabolism of atrazine by the soluble fraction (105,000) from chicken liver homogenates. J. Agric. Food Chem. **27:**300–302.
17. **Giardina, M. C., M. T. Giardi, and G. Filacchioni.** 1980. 4-Amino-2-chloro-1,3,5-triazine: a new metabolite of atrazine by a soil bacterium. Agric. Biol. Chem. **44:**2067–2072.
18. **Gojmerak, T., and J. Kniewald.** 1990. Identification of atrazine residue and its biodegradation products in turkey tissues. Vet. Glas. **44:**135–143.
19. **Gysin, H., and E. Knuesli.** 1960. Chemistry and herbicidal properties of triazine derivatives. Adv. Pest Control Res. **3:**289–358.
20. **Jessee, J. A., R. E. Benoit, A. C. Hendricks, G. C. Allen, and J. L. Neal.** 1983. Anaerobic degradation of cyanuric acid, cysteine, and atrazine by a facultative anaerobic bacterium. Appl. Environ. Microbiol. **45:**97–102.
21. **Kello, D.** 1989. WHO drinking water quality guidelines for selected herbicides. Food Addit. Contam. **6**(Suppl. 1)**:**S79–S85.
22. **Khan, S. U., T. S. Foster, and M. H. Akhtar.** 1979. Metabolism of atrazine by the soluble fraction (105,000) from chicken liver homogenates. Pestic. Sci. **10:**460.
23. **Koplin, D. W., and S. J. Kalkhohh.** 1993. Atrazine degradation in a small stream in Iowa. Environ. Sci. Technol. **27:**134–139.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London) **227:**680–685.
25. **Mandelbaum, R. T., D. L. Allan, and L. P. Wackett.** 1995. Isolation and characterization of a *Pseudomonas* sp. that mineralizes the *s*-triazine herbicide atrazine. Appl. Environ. Microbiol. **61:**1451–1457.
26. **Mandelbaum, R. T., L. P. Wackett, and D. L. Allan.** 1993. Mineralization of the *s*-triazine ring of atrazine by stable bacterial mixed cultures. Appl. Environ. Microbiol. **59:**1695–1701.
27. **Mandelbaum, R. T., L. P. Wackett, and D. L. Allan.** 1993. Rapid hydrolysis of atrazine to hydroxyatrazine by soil bacteria. Environ. Sci. Technol. **27:** 1943–1946.
28. **Mulbry, W. W.** 1994. Purification and characterization of an inducible *s*-triazine hydrolase from *Rhodococcus corallinus* NRRL B-15444R. Appl. Environ. Microbiol. **60:**613–618.
29. **Nagy, I., F. Compernolle, K. Ghys, J. Vanderleyden, and R. de Mot.** 1995. A single cytochrome P-450 system is involved in degradation of the herbicides EPTC (*s*-ethyl dipropylthiocarbamate) and atrazine by *Rhodococcus* sp. strain NI86/21. Appl. Environ. Microbiol. **61:**2056–2060.

30. **Parsons, D. W., and J. M. Witt.** 1988. Pesticides in ground water in the United States of America: a report of a 1988 survey of state lead agencies. Oregon State University Agricultural Chemistry Department, Corvallis.
31. **Pick, F. E., L. P. van Dyk, and E. Botha.** 1992. Atrazine in ground and surface water in maize production areas of Transvaal, South Africa. Chemosphere **25:**335–341.
32. **Radosevich, M., S. J. Traina, Y. L. Hao, and O. H. Tuovinen.** 1995. Degradation and mineralization of atrazine by a soil bacterial isolate. Appl. Environ. Microbiol. **61:**297–302.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Seiler, A., P. Brenneisen, and D. H. Green.** 1992. Benefits and risks of plant protection products—possibilities of protecting drinking water: case atrazine. Water Supply **10:**31–42.
35. **Shao, Z., and R. Behki.** 1995. Cloning of the genes for degradation of the herbicides EPTC (*s*-ethyl dipropylthiocarbamate) and atrazine by *Rhodococcus* sp. strain TE1. Appl. Environ. Microbiol. **61:**2061–2065.
36. **Shao, Z. Q., W. Seffens, W. Mulbry, and R. M. Behki.** 1995. Cloning and expression of the *s*-triazine hydrolase gene (*trzA*) from *Rhodococcus corallinus* and development of *Rhodococcus* recombinant strains capable of dealkylating and dechlorinating the herbicide atrazine. J. Bacteriol. **177:**5748–5755.
37. **Shigler, V., J. Powlowski, and V. Marklund.** 1992. Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600. J. Bacteriol. **174:**711–724.
38. **Shimabukuro, R. H.** 1967. Atrazine metabolism and herbicidal selectivity. Plant Physiol. **43:**1925–1930.
39. **Staden, R.** 1984. Computer methods to locate signals in nucleic acid sequences. Nucleic Acids Res. **12:**505–519.
40. **Steiert, J. G., and R. L. Crawford.** 1986. Catabolism of pentachlorophenol by a *Flavobacterium* sp. Biochem. Biophys. Res. Commun. **141:**825–830.
41. **Xun, L., and C. S. Orser.** 1991. Purification and properties of pentachlorophenol hydroxylase, a flavoprotein from *Flavobacterium* sp. strain ATCC 39723. J. Bacteriol. **173:**4447–4453.
42. **Yanze-Kontchou, C., and N. Gschwind.** 1994. Mineralization of the herbicide atrazine as a carbon source by a *Pseudomonas* strain. Appl. Environ. Microbiol. **60:**4297–4302.