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# Dechlorination of Atrazine by a *Rhizobium* sp. Isolate

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**A *Rhizobium* sp. strain, named PATR, was isolated from an agricultural soil and found to actively degrade the herbicide atrazine. Incubation of PATR in a basal liquid medium containing 30 mg of atrazine liter$^{-1}$ resulted in the rapid consumption of the herbicide and the accumulation of hydroxyatrazine as the only metabolite detected after 8 days of culture. Experiments performed with ring-labeled [$^{14}$C]atrazine indicated no mineralization. The enzyme responsible for the hydroxylation of atrazine was partially purified and found to consist of four 50-kDa subunits. Its synthesis in PATR was constitutive. This new atrazine hydrolase demonstrated 92% sequence identity through a 24-amino-acid fragment with atrazine chlorohydrolase AtzA produced by *Pseudomonas* sp. strain ADP.**

Atrazine, an *s*-triazine herbicide, is extensively used for the selective and nonselective control of weeds worldwide. Although this compound is only moderately persistent in the environment, with a half-life of 1 to 12 months (31), its intensive use has led to significant contamination of ground- and surface water in several countries (4, 5, 15, 17, 21). As this pollutant may be a hazard to human health, much attention has been paid to its fate in soil and to the chemical and biological processes involved in its degradation (13).

Atrazine is generally considered to be more resistant to enzymatic attack from environmental microorganisms than less heavily substituted and nonchlorinated *s*-triazine analogs, like melamine or cyanuric acid, that are used as nitrogen and carbon sources by several microbial species (6, 7, 8, 19). Several microorganisms isolated as pure or mixed cultures from treated soils have been reported to promote limited degradation of the atrazine molecule (1, 2, 3, 11, 16, 25, 26, 33). These isolates usually remove the side chains by N dealkylation and, subsequently, use the ethyl and isopropyl carbons via oxidative phosphorylation. Only a few microorganisms can enzymatically break down the *s*-triazine ring, leading to the complete mineralization of the herbicide. Several authors have succeeded in isolating mixed consortiums or pure cultures that could mineralize atrazine (22, 24, 28, 35). Hydroxyatrazine, the dehalogenated derivative of atrazine, was identified as the first intermediate in the catabolism of one of these isolates, *Pseudomonas* sp. strain ADP (9, 24). These findings indicate that dechlorination of *s*-triazine compounds can be mediated biologically and is not due solely to chemical processes, as previously thought (14, 20). Two enzymes that are able to dehalogenate *s*-triazines have been characterized: an *s*-triazine hydrolase from a *Rhodococcus corallinus* strain (27) and an atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP (10). This paper describes a new bacterial isolate that produces a hydrolase enzyme involved in the transformation of atrazine to hydroxyatrazine.

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## MATERIALS AND METHODS

**Chemicals.** Atrazine was obtained from Riedel-de-Haën (SISAF Sotrachem, La Plaine St. Denis, France); hydroxyatrazine and other *s*-triazine compounds were provided by CIL (Ste. Foy-la-Grande, France). [Ring-U-$^{14}$C]atrazine was purchased from Sigma (St. Quentin Fallavier, France). The nomenclature used for the *s*-triazine molecules was as previously described by Cook (6), where C, E, I, B, and O designate chloro, ethylamino, isopropylamino, terbutylamino, and hydroxy substituents, respectively, and T designates the *s*-triazine ring. The trivial names and abbreviations of the *s*-triazine molecules used in the study are as follows: atrazine (CIET), deethylatrazine (CIAT), deisopropylatrazine (CEAT), dealkylatrazine (CAAT), hydroxyatrazine (OIET), deethylhydroxyatrazine (OIAT), deisopropylhydroxyatrazine (OEAT), simazine (CEET), propazine (CIIT), and terbutylazine (CEBT).

**Soil samples and growth media.** Eleven soil specimens were collected from agricultural fields in the east of France that had been treated with atrazine preparations (Doubs and Jura regions). The soil samples were cultured in liquid minimal salts nitrogen medium (BMN) supplemented with 50 mg of atrazine liter$^{-1}$ (BMNA50) (1). The *Rhizobium* strain PATR was isolated on a solid minimal medium containing 200 mg of finely dispersed atrazine liter$^{-1}$ (BMNA200). M9 minimal medium (30) containing no $NH_4Cl$ and supplemented with 30 mg of atrazine liter$^{-1}$ and 2 g of glucose liter$^{-1}$ was used for cultures with radiolabeled atrazine (MGA medium). Other bacterial cultures were performed in Mueller-Hinton medium or tryptone yeast extract (TY) (Becton Dickinson, Meylan, France).

**Enrichment cultures and identification of the bacterial isolate.** BMNA50 (20 ml) was inoculated with 5 g of soil sample and incubated at 30°C without shaking. Aliquots were subcultured (0.4 ml into 20 ml of fresh medium) every 2 weeks for 2 months, and aliquots of the final subcultures were plated on BMNA50 agar plates. The colonies developing on BMNA50 were repeatedly streaked on BMNA200 and on Mueller-Hinton plates to check for purity. Atrazine degradation was suspected when colonies on BMNA200 plates were surrounded by a clear halo (24). A bacterial isolate, designated PATR, was selected for further analysis but could not be identified by conventional methods based on substrate specificity and biochemical reactions (Api20NE kit; bioMérieux SA, Marcy l'Etoile, France). The genus of PATR was subsequently determined by amplifying and sequencing 16S rRNA genes (34) in the laboratory of Patrick Grimont (Pasteur Institute, Paris, France).

**Incubation with [ring-U-$^{14}$C]atrazine.** An overnight culture of PATR was diluted 50-fold in MGA medium containing 25 nCi of [ring-U-$^{14}$C]atrazine ml$^{-1}$, and the cells were incubated at 30°C with shaking. Aliquots were removed every 3 h for liquid scintillation counting (Aquasafe 300 plus; Zinsser Analytic) to measure radioactivity in (i) the total culture (bacteria and medium), (ii) the culture supernatant obtained after centrifugation for 10 min at 10,000 × *g*, and (iii) the cell pellet. To count cell-associated radioactivity, 0.5 ml of culture was centrifuged (10,000 × *g* for 10 min) in a Millipore Ultrafree MC filtration unit (St. Quentin, France). The filter was washed with 1 ml of distilled water and placed in the scintillation cocktail for counting. All experiments were performed in triplicate.

**Analysis of atrazine metabolites.** Atrazine metabolites were detected and identified by reverse-phase high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS), using atmospheric pressure chemical ionization (Finnigan TSQ 700). Separation was done at a 1-ml min$^{-1}$ flow rate on a $C_{18}$-nucleosil 5-μm column (250 by 4.6 mm) with a water-acetonitrile

gradient (100:0 to 20:80 [vol/vol]). Aliquots of filtered culture medium (50 μl) were taken at regular intervals from 0 to 200 h and injected into the column. Metabolites were identified by recording full mass spectra and by monitoring the $MH^+$ ions of all possible metabolites (reconstruction of ion chromatograms). The HPLC separations were also monitored by UV absorption with a diode array detector (Applied Biosystems 1000S).

The metabolite was confirmed as hydroxyatrazine by electron impact gas chromatography (GC)-MS (VG instruments; Autospec) of its trimethylsilyl derivative (bis silyl trifluoroacetamide plus 1% trimethylchlorosilane, overnight at 80°C). The spectrum showed the molecular ion (*m/z*, 269); loss of a methyl radical; and losses of propene, ethylene, or both, from $M^+$ or from $(M - 15)^+$.

Atrazine and hydroxyatrazine were quantified by electrospray (VG instruments Trio 2000). CEET (2 ng) was added to each aliquot of culture medium (100 μl) as the internal standard, and the whole was diluted with 80 μl of acetonitrile containing 4% formic acid. Ten microliters of this mixture was directly injected into the flow of the electrospray source (water-acetonitrile, 1:1, containing 2% formic acid, 10 μl $min^{-1}$). Multiple-ion monitoring was used for detection, and ion currents of *m/z* 202 and 204 (CEET), *m/z* 216 and 218 (atrazine), and *m/z* 198 (hydroxyatrazine) were recorded. Calibration curves were prepared with authentic standards and gave linear relationships when the atrazine concentration was plotted against the ion intensity ratio (*m/z* 216)/(m/z 202) or when the hydroxyatrazine concentration was plotted against the intensity ratio (*m/z* 198)/(*m/z* 202), within the relative proportion range, analyte-CEET 1:10 to 10:1.

Culture supernatants containing radiolabeled atrazine were analyzed by HPLC and thin-layer chromatography (TLC), using known atrazine derivatives as references. HPLC was performed with a Waters system (Wisp 712, pumping system 600E) equipped with a Waters UV detector 484 set at 230 nm and connected to an LB.506.C1 radioactivity monitor (Berthold). The *s*-triazine compounds were separated on an S5-ODS2 column (SupRs Classic, 250 by 4.6 mm) eluted with an acetonitrile gradient (5 to 70%) in 0.01 M $KH_2PO_4$, pH 2.1, as previously described by Rustum et al. (29). TLC analysis was done on precoated silica gel $60F_{254}$ plates (Merck, Nogent-sur-Marne, France) developed in solvent I (chloroform-acetone-acetic acid, 100:5:2 [vol/vol/vol]) to separate atrazine from its dealkylated derivatives or in solvent II (chloroform-ethanol-acetic acid, 100:10:10 [vol/vol/vol]) to resolve the hydroxylated compounds. The chromatograms were scanned with an automatic TLC linear analyzer (Tracemaster 20; Berthold) to measure the radioactivity in each spot.

**Estimation of $^{14}CO_2$.** Strain PATR was incubated in MGA medium containing [ring-U-$^{14}$C]atrazine as described above, except that the flasks were hermetically sealed. The flasks were flushed with air every day, and the exhaust gases were passed through two vials in series containing 2 ml of 2 N NaOH to trap $^{14}CO_2$. The amount of radioactivity in the NaOH traps was measured by liquid scintillation counting.

**Partial purification of atrazine hydrolase.** Cells from a stationary-phase culture in TY were collected by centrifugation (5,000 × *g* for 15 min) and washed with 30 mM phosphate buffer (pH 7.4). The cells were disrupted by sonication, and the debris was removed by centrifugation (10,000 × *g* for 30 min). Proteins were precipitated from the supernatant between 20 and 50% (wt/vol) ammonium sulfate. The pellet was resuspended in 30 mM phosphate buffer (pH 7.4) and dialyzed extensively against the same buffer. The preparation was loaded onto a DE52 column (Whatman, Maidstone, England) equilibrated with 30 mM phosphate buffer (pH 7.4). The column was washed with the same buffer to remove unbound material, and the bound proteins were then eluted with a concentration gradient of NaCl (0 to 0.5 M). The enzymatically active fractions were pooled and applied to a Sephacryl S-200HR column (Pharmacia, Uppsala, Sweden) equilibrated and eluted with 30 mM phosphate buffer (pH 7.4) containing 0.25 M NaCl. Fractions in which the hydrolase activity was detected were concentrated with a Centriprep 10 concentrator (Amicon, Beverly, Mass.). Protein content was measured with the BCA reagent (Pierce Chemical Co, Rockford, Ill.) calibrated with bovine serum albumin. Column fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the proteins were stained with silver or Coomassie brilliant blue R250 (30). Nondenaturing gel electrophoresis was performed by omitting SDS from all buffers. The nondenaturing gels were stained with Coomassie brilliant blue or overlaid with a thin layer of agar containing a microsuspension of atrazine. The layered gels were incubated at 30°C to detect the formation of a clear zone due to atrazine transformation at the site of atrazine hydrolase.

**Atrazine hydrolase assay.** Atrazine hydrolase activity was detected by TLC. Lysate proteins (100 μg) were incubated for 1 h at 55°C with 1 mM atrazine in 50 mM Tris-HCl (pH 8.2) containing 0.1 mM $MgSO_4$; 10-μl samples were analyzed by TLC.

A rapid assay adapted from that previously described by Mulbry (27) was used to measure the atrazine hydrolase activity in crude cell extracts. Typically, 10 μg of lysate proteins was incubated in 50 mM Tris-HCl (pH 8.2) containing 0.1 mM $MgSO_4$ and 25 μM atrazine. The enzymatic conversion of atrazine to hydroxyatrazine was monitored spectrophotometrically by measuring the decrease in absorbance at 225 nm. Optimal conditions of pH and temperature for enzyme activity were determined in the presence of various divalent cations added at a final concentration of 0.1 mM each. The specific enzyme activity was expressed in nanomoles of atrazine transformed per minute per milligram of protein.

**Enzyme induction.** The induction of atrazine hydrolase synthesis by atrazine was studied mainly as previously described by Mulbry (27). Cells grown to stationary phase in TY were washed, resuspended in MGA medium or in the same medium containing $NH_4Cl$ instead of atrazine, and incubated at 30°C for 24 h. The cells were collected by centrifugation, sonicated, and assayed for atrazine hydrolase activity as described above.

**Protein microsequencing.** The sequence of a 24-amino-acid internal peptide of atrazine hydrolase was determined after cleavage of the purified protein by lysine C endoprotease (Microsequencing Laboratory, Pasteur Institute, Paris, France).

## RESULTS

**Isolation and characterization of PATR.** Enrichment cultures of one of the 11 soil specimens yielded a gram-negative bacterium that produced clear zones around its colonies on minimal agar plates supplemented with atrazine. The presence of these halos was attributed to the metabolization of atrazine to soluble products. The isolate, designated PATR, was identified as a *Rhizobium* sp. by 16S rRNA analysis.

**Degradation of atrazine to hydroxyatrazine.** The enzymatic cleavage of atrazine by PATR was confirmed by incubating the cells in MGA medium containing [ring-U-$^{14}$C]atrazine. Culture samples were analyzed by HPLC and TLC, and the re-

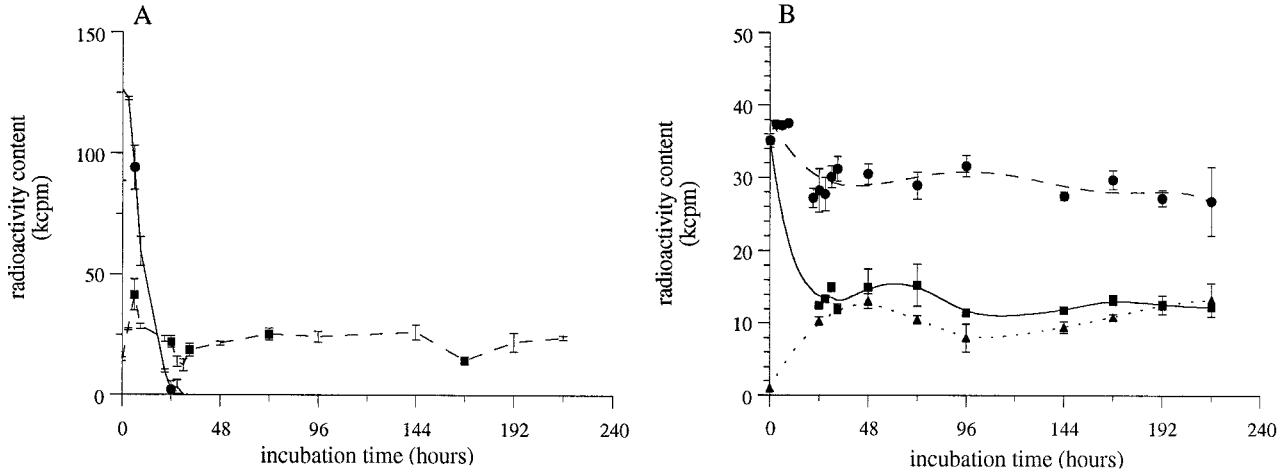


FIG. 1. (A) Changes in the concentrations of atrazine (●) and hydroxyatrazine (■) in PATR culture supernatants. Radioactivity was measured with a TLC analyzer. (B) Changes in radioactivity in the total culture (●), supernatants (■), and cells (▲), during incubation with [ring-U-$^{14}$C]atrazine.

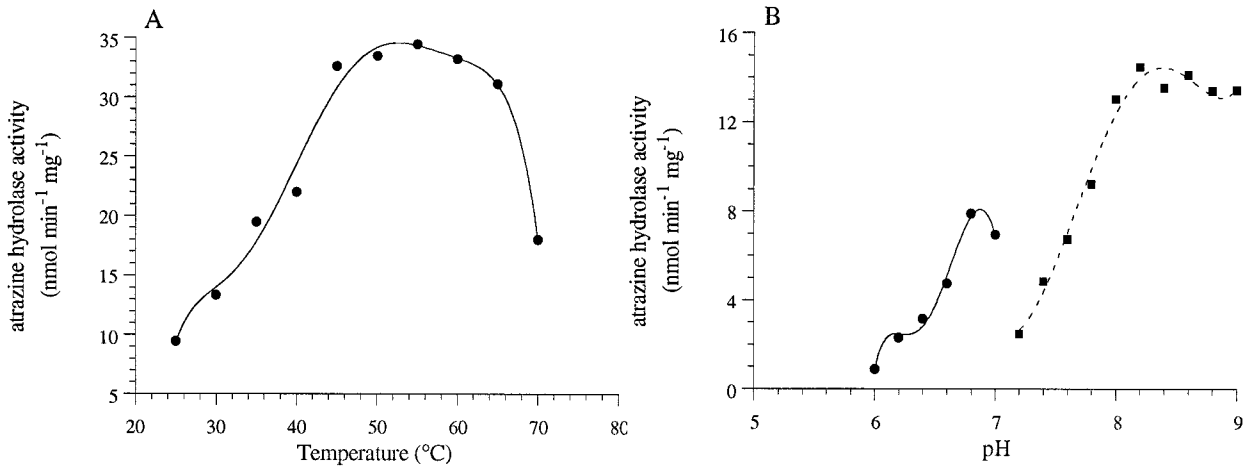


FIG. 2. Optimal temperature (A) and pH (B) conditions for atrazine hydrolase activity. Measurements were made in phosphate buffer (●) and in Tris-HCl buffer (■).

sultant chromatograms were compared with those of *s*-triazine standards: atrazine, hydroxyatrazine, CEAT, CIAT, CAAT, OIAT, and OEAT. Atrazine was no longer detected in the culture medium after 24 h of incubation. The disappearance of the substrate was correlated with the accumulation of a single radioactive compound that remained unaltered after 8 days of incubation (Fig. 1A). Quantification of atrazine by electrospray MS in aliquots taken at regular intervals of time confirmed its decrease and its almost complete disappearance after 8 days. HPLC-MS indicated that the metabolite formed was hydroxyatrazine. It was identified by its retention volume, its molecular weight (HPLC-MS), and by electron impact GC-MS of its trimethylsilyl derivative. No other metabolite was detected by MS or by UV detection.

Radioactivity was measured every day for 8 days in (i) the whole aliquot, (ii) the supernatant, and (iii) the bacterial cell pellet to determine whether PATR mineralized atrazine. Total radioactivity in the cultures remained almost constant over time, with negligible $^{14}CO_2$ release (<1,000 cpm). The amount of radioactivity in the supernatant decreased within the first 24 h (by 55%) but then stabilized for the rest of the incubation period. In parallel, 36% of the total radioactivity was found to be cell associated after 24 h (Fig. 1B).

**Characterization of atrazine hydrolase.** Twenty micrograms of PATR lysate proteins produced a clear zone on atrazine agar plates after incubation at 30°C for 24 h. TLC analysis showed that the lysate, like the whole cells, rapidly transformed atrazine into hydroxyatrazine, while boiled lysate (3 min at 100°C) or lysate treated with proteinase K (1 h at 37°C) did not degrade the herbicide. The atrazine hydrolase enzyme was active over a broad range of pHs and temperatures, with optima at 8.2 and 55°C, respectively (Fig. 2). No cofactors were required for activity. $Co^{2+}$ was the only divalent cation tested that stimulated hydrolase activity (threefold). In contrast, $Cu^{2+}$ and $Zn^{2+}$ strongly inhibited the enzyme, while $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ had no effect on its activity. The ability of atrazine hydrolase to dechlorinate other *s*-triazines was determined by TLC. The enzyme hydrolyzed CEET, CIAT, and CEBT but not CIIT, indicating that two isopropyl groups on the *s*-triazine ring prevent hydroxylation. We also tested atrazine as an inducer of atrazine hydrolase synthesis. There was no difference in enzyme activities between the cells incubated with atrazine and the controls. These data indicate that atrazine hydrolase is constitutively expressed in PATR and is not induced by atrazine.

Atrazine hydrolase was purified from crude cell extracts by $(NH_4)_2SO_4$ precipitation, DE52 anion-exchange chromatography, and gel filtration on Sephacryl S-200HR (Table 1). The final preparation contained one major protein band migrating at about 50 kDa on an SDS–12% polyacrylamide gel (Fig. 3). The native molecular size of atrazine hydrolase was estimated on a nondenaturing polyacrylamide gel to be about 200 kDa. This suggests that the enzyme is a tetramer of identical 50-kDa subunits.

The enzyme was unambiguously identified by analysis of the purified fraction in a nondenaturing polyacrylamide gel overlaid with a microsuspension of atrazine in agar. A clear halo appeared at the place of the main protein band. Proteins separated under denaturing conditions did not produce such a clear zone.

**Protein microsequencing and sequence comparison.** A 24-amino-acid internal sequence was determined after treatment of the purified enzyme with lysine C endoprotease. A search for homologies with other proteins in several databases indicated that the atrazine hydrolase fragment had 92% identity with a sequence of atrazine chlorohydrolase (AtzA) from *Pseudomonas* sp. strain ADP (10) and 54% identity with a sequence of chlorohydrolase (TrzA) from *R. corallinus*, an

TABLE 1. Partial purification of the atrazine hydrolase from PATR

| Purification step | Total protein (mg) | Total activity (nmol min$^{-1}$) | Sp act (nmol min$^{-1}$ mg protein$^{-1}$) | Recovery (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude extract | 100 | 90 | 0.9 | 100 | 1 |
| $(NH_4)_2SO_4$ (20–50%) | 20 | 48 | 2.4 | 53.3 | 2.6 |
| DE52 | 3 | 43.8 | 14.6 | 48.7 | 15.8 |
| S-200HR | 0.8 | 13 | 16.2 | 14.4 | 17.6 |

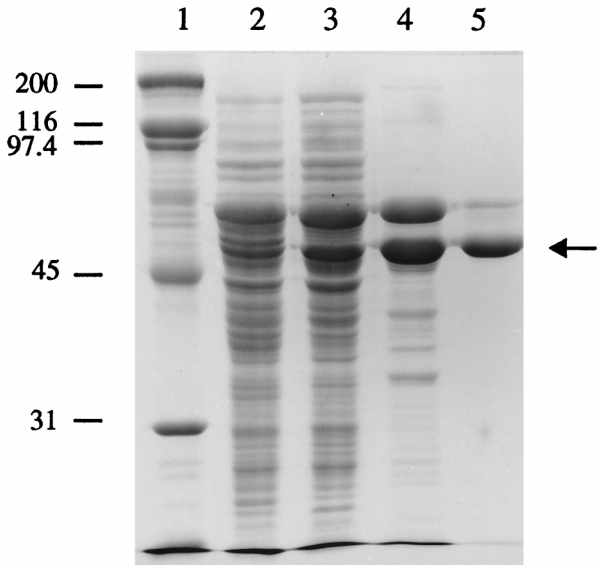


FIG. 3. Purification of atrazine hydrolase. Molecular mass markers (in kilodaltons) (lane 1), crude extract (lane 2), ammonium sulfate fraction (20 to 50%) (lane 3), DE52 fraction (lane 4), and Sephacryl S200-HR fraction (lane 5) were separated on an SDS-polyacrylamide gel (12% polyacrylamide). The atrazine hydrolase band is indicated by an arrow.

enzyme involved in the dehalogenation of CEAT (32) (Table 2).

## DISCUSSION

A new atrazine-degrading bacterium identified as a *Rhizobium* sp. by 16S rRNA analysis has been isolated from an agricultural soil treated with the herbicide. Enzymatic transformation of atrazine was initially detected by a zone-clearing assay on minimal salt medium containing atrazine as the sole source of carbon and/or nitrogen. Small colonies of *Rhizobium* sp. strain PATR surrounded by a clear halo were easily distinguished after several days of incubation, but their growth was extremely slow, suggesting that atrazine is not a growth substrate for the bacterium. $^{14}C$ evolution experiments with [ring-U-$^{14}C$]atrazine confirmed the absence of mineralization of the herbicide. On the other hand, long-term incubation of PATR cells with atrazine resulted in accumulation of hydroxyatrazine as the unique metabolite detected by TLC or HPLC. Atrazine dechlorination thus appears to be the major atrazine-degrading activity expressed by PATR. This conclusion is consistent with the formation of clear zones on atrazine agar plates, since hydroxylation is known to significantly increase the water solubility of the herbicide (13). In the experiments with radiolabeled atrazine, a large fraction (36%) of the ring carbon atoms was found to be associated with the bacterial cells. It could therefore be argued that intermediates other than hydroxyatrazine are formed and incorporated into cell components. For example, cyanuric acid (OOOT) is known to be used as an intermediate in nucleic acid synthesis and to support growth of uracil-requiring *E. coli* mutants (18). The lack of evidence for further metabolism past hydroxyatrazine in PATR, however, supports the idea that the ring carbon atoms were not incorporated into, but associated with, cell constituents.

TABLE 2. Comparison of a 24-amino-acid sequence from PATR atrazine hydrolase and sequences of other triazine hydrolases[a]

| | |
|---|---|
| PATR atrazine hydrolase | .....T**SQVVSNAYLGSGV**Q**PVPEMVERG** |
| *Pseudomonas* sp. hydrolase AtzA | ....A**SQVVSNAYLGSGVAPVPEMVERG** |
| *R. corallinus s*-triazine hydrolase TrzA | .....S**T**Q**PVSN**S**YL**AA**G**I**APVPEM**LAH**G** |

[a] Identical amino acid residues are indicated in boldface type.

The enzyme catalyzing atrazine dechlorination in PATR was partially purified and found to contain four 50-kDa subunits as *s*-triazine hydrolase from *R. corallinus* (27). A search for amino acid similarities with other proteins revealed that the hydrolase fragment of PATR enzyme has 92% identity with an internal sequence of atrazine chlorohydrolase (AtzA) from *Pseudomonas* sp. strain ADP (10). These two enzymes thus appear to be closely related. Their substrate specificity is not, however, strictly identical, since hydrolase from PATR, but not that from ADP, dechlorinates CEBT. Interestingly, both atrazine hydrolases also exhibit significant sequence homology with *s*-triazine hydrolase (TrzA) from *R. corallinus*, an enzyme which is active on CEAT and CIAT but not on atrazine and CEET and which promotes the deamination of a number of molecules such as melamine, CAAT, and pyrimidine compounds (27). Melamine was not deaminated by PATR hydrolase (data not shown) or by AtzA (10).

Atrazine dechlorination has been reported to occur less frequently than N dealkylation reactions in atrazine-degrading microorganisms (2, 23, 28). However, hybridization experiments using a DNA probe derived from the gene encoding AtzA in *Pseudomonas* sp. strain ADP suggest that homologous genes might be widespread in nature and could account for the formation of hydroxyatrazine in contaminated soils (9). Because of their frequent occurrence in fertilized soils, members of the genus *Rhizobium*, such as PATR, are likely to play an important role in the biological degradation of atrazine.

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