APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1451–1457 Vol. 61, No. 4
0099-2240/95/$04.00+0

Isolation and Characterization of a *Pseudomonas* sp. That Mineralizes the *s*-Triazine Herbicide Atrazine

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Received 29 August 1994/Accepted 23 January 1995

A bacterium that was capable of metabolizing atrazine at very high concentrations (>1,000 ppm) was isolated from a herbicide spill site. The organism was differentiated by observing clearing zones on indicator agar plates containing 1,000 ppm atrazine. Detailed taxonomic studies identified the organism as a *Pseudomonas* sp., designated ADP, that was dissimilar to currently known species. *Pseudomonas* sp. strain ADP metabolized atrazine as its sole nitrogen source. Nongrowing suspended cells also metabolized atrazine rapidly; for example, 9×10^9 cells per ml degraded 100 ppm of atrazine in 90 min. Atrazine was metabolized to hydroxyatrazine, polar metabolites, and carbon dioxide. When uniformly ring-labeled [^{14}C]atrazine was used, 80% of the radioactivity was liberated as $^{14}CO_2$. These data indicated the triazine ring was completely mineralized. The isolation and characterization of *Pseudomonas* sp. strain ADP may contribute to efforts on atrazine bioremediation, particularly in environments containing very high pesticide levels.

Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine] is the predominant member of a broad class of triazine herbicides used for controlling broad-leaved weeds. Approximately 800 million lb (363 million kg) was applied in the United States between 1980 and 1990 (16). Atrazine is moderately persistent in soil, with reported half-lives ranging from 4 to 57 weeks (5, 7). The mobility of atrazine in soil has contributed to the contamination of groundwater (2, 4, 13, 32). Because of its widespread usage, atrazine concentrations in groundwater and surface water frequently exceed the 3-ppb health advisory level set by the U.S. Environmental Protection Agency (36).

The prevalence of atrazine in the environment has stimulated investigations into the biodegradation of the class of compounds containing an *s*-triazine ring (8, 14). Enrichment culture techniques have provided bacteria for investigations into the biochemistry and molecular biology underlying *s*-triazine metabolism. For example, a *Rhodococcus* strain biosynthesizes a halidohydrolase that is active with certain chlorinated *s*-triazine compounds (10, 27). A *Pseudomonas* sp. that metabolizes melamine contains an inducible set of genes that encode the enzymes of a melamine-degradative pathway (11, 12). These two organisms and most others isolated to metabolize *s*-triazines fail to metabolize atrazine. There are a limited number of reported pure cultures that metabolize atrazine. In 1986, a *Pseudomonas* sp. was reported to grow on atrazine over a 30-day period via oxidation of the alkyl side chains (3). Recently, bacterial isolates that mineralize the *s*-triazine ring of atrazine have been described (26, 33, 38), but there is still little information available on the genes, enzymes, and metabolites involved.

In a previous study, we described a mixed bacterial culture, enriched from a herbicide spill site, that metabolized atrazine as its sole nitrogen source (24). The use of *s*-triazines as the sole nitrogen source had been an established enrichment strategy but had previously failed to yield atrazine-metabolizing bacteria (8, 9). Atrazine metabolism by the mixed culture was relatively rapid and complete, suggesting that more-detailed studies of this system were warranted. In the present work, a method is described that should have general application in the isolation of other atrazine-metabolizing bacteria in pure culture. The method was applied to the mixed culture (24) and yielded an atrazine-metabolizing *Pseudomonas* sp. denoted here as ADP. *Pseudomonas* sp. strain ADP used atrazine as the sole source of nitrogen but metabolized much more than required for its nitrogen assimilation. Media containing 1,000 ppm of atrazine were cleared, and 80% of the *s*-triazine ring carbon atoms were transformed to carbon dioxide. *Pseudomonas* sp. strain ADP yielded stable cell-free atrazine-metabolizing activity. These data indicate that this is an important new strain that will be useful for better understanding the microbiology and biochemistry of bacterial atrazine metabolism.

MATERIALS AND METHODS

Materials. Atrazine (99.6% chemical purity) was purchased from Chem Service, West Chester, Pa. Uniformly ring-labeled [^{14}C]atrazine (7.8 mCi/mmol; 99.6% radiochemical purity) was purchased from Sigma Radiochemicals, St. Louis, Mo. Authentic samples of simazine, desisopropylatrazine, desethylatrazine, desethyldesisopropylatrazine, hydroxyatrazine, desethylhydroxyatrazine, desisopropylhydroxyatrazine, and desethyldesisopropylhydroxyatrazine were a gift from Ciba Geigy Corp., Greensboro, N.C. Individual 100-ppm stock solutions of authentic atrazine and metabolite standards were prepared in methanol and stored at 4°C. All other chemicals used were of reagent grade or better.

Growth media. The liquid growth medium (atrazine medium) used was described previously (24). It contained 0.1% (wt/vol) sodium citrate as the carbon source and atrazine as the only nitrogen source at a final concentration of 100 ppm (0.46 mM) unless stated otherwise. The solid medium used to indicate atrazine degradation contained the same mineral salts as the liquid medium, 1,000 ppm of atrazine, and 2% (wt/vol) Noble agar (Difco Laboratories, Detroit, Mich.). Atrazine was added from a highly concentrated stock solution (500 mg/ml in methanol) that was sonicated to reduce the particle size of the atrazine crystals. The short sonication process did not cause any modification of the molecule, as tested by high-pressure liquid chromatography (HPLC) analysis. Agar plates were cloudy as a result of particulate atrazine in the medium, enabling visual inspection of clearing zones around atrazine-degrading colonies. King's medium B was used as originally described (19). Triple sugar iron agar (Oxoid, Basingstoke, England) and tryptic soy agar (Difco) were obtained as premixed media and used as specified by the manufacturers.

Pure-culture isolation. Mixed bacterial culture LFB6 (24) was cultured for 3 days in 500-ml Erlenmeyer flasks containing 300 ml of atrazine medium and grown to an optical density at 600 nm (OD_{600}) of >1.0 without shaking. The cells

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were harvested by centrifugation at 6,000 × *g* and 4°C for 20 min and washed twice with sterile 0.1 N sodium phosphate buffer (pH 7.0). The pellet was resuspended in sterile buffer, serially diluted in 10-fold increments, and plated on atrazine (100 ppm) solid medium. After 3 days of incubation at 30°C, colonies showing good growth (colony diameter larger than 2 to 3 mm) were transferred to a solid atrazine medium containing 1,000 ppm of atrazine. The plates were incubated for several days at 30°C and periodically examined for clearing zones. A colony showing extensive clearing was selected. After several transfers onto atrazine medium, trypic soy agar, and King's B medium plates, it was determined to be pure. Purity was also tested by transmission electron microscopy (Hitachi H-600 microscope) with bacteria from a single 48-h colony growing on atrazine medium. All individual bacteria were similar morphologically.

Bacterial identification. Most biochemical tests were performed with the Biolog identification system (Biolog, Hayward, Calif.) with manual reading of the plates. The pure culture was grown on atrazine solid medium and transferred to tryptic soy agar medium as specified in the Biolog manual. The plates were incubated at 30°C in the dark, and readings were taken 24 h after inoculation. Additional biochemical tests were performed. Triple sugar iron agar slants were used to determine fermentation of glucose. Oxidase activity was determined by the modified Kovac oxidase test (15). King's medium B (19) was used for testing pyoverdin production.

For sequencing 16S rRNA, bacterial cell lysis and total RNA isolation from *Pseudomonas* strain ADP and *Pseudomonas citronellolis* ATCC 13674 were performed with a Bead-beater as described previously (35). The 16S rRNA in bulk cellular RNA preparations was used for sequencing analysis without further purification. Eight synthetic 16S rRNA-specific oligonucleotide primers, which complement conserved regions in the 16S rRNA of *Escherichia coli* (30), were used as specified by Giovannoni et al. (17). The primer sequences were elongated by use of a reverse transcriptase (22), and the resulting DNA sequences were determined by the dideoxynucleotide-sequencing method (34). Sequences were aligned on a VAX 8600 computer by using published programs (29) and compared with sequences in GenBank.

Flagellar morphology was determined with a Hitachi H-600 transmission electron microscope. Bacteria were grown to late log phase in atrazine liquid medium, gently washed twice with 100 mM phosphate buffer (pH 7.0), and negatively stained with uranyl acetate.

Determination of bacterial growth. Cell growth was measured spectrophotometrically by measuring the OD_{600} in a DU-70 spectrophotometer (Beckman Instruments, Fullerton, Calif.). The protein content of the culture was determined after cell lysis in 0.1 N NaOH at 80°C for 1 h. A bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, Ill.) with a bovine serum albumin standard was used.

Atrazine metabolism by resting-cell cultures. Exponential-phase cells grown in atrazine liquid medium were harvested by centrifugation at 18,000 × *g* for 30 min, washed twice with 50 mM phosphate buffer (pH 7.2), and resuspended in fresh buffer to an A_{600} of 5.0. This corresponds to 10^{10} bacteria and 1.5 mg of protein per ml. Uniformly ring-labeled [^{14}C]atrazine was added to yield a suspension of 100 ppm and 25 nCi/ml of medium. At various time points, 1 ml was removed and extracted with an equal volume of ethyl acetate. The radioactivity in each phase was determined with a Beckman LS 6800 scintillation counter, with channels set to 0 to 670 and 250 to 670 for background and sample readings, respectively. The degradation of atrazine was measured as the disappearance of radioactivity (atrazine) from the ethyl acetate phase and the appearance of transient nonvolatile metabolites in the aqueous phase. This was found to be consistent with atrazine disappearance and metabolite formation as determined by HPLC.

To determine metabolite and carbon dioxide formation, 20 ml of 50 mM phosphate buffer (pH 7.2) was transferred to 250-ml biometric Erlenmeyer flasks. Cells obtained from a culture grown on atrazine were added to yield 1.3 × 10^{10} cells per ml, and the flasks were stoppered after being flushed with oxygen gas. Atrazine was added to 100 ppm and 4 μCi. The flasks were fitted with two air-tight syringes equipped with 22-gauge needles reaching the growth medium to sample the liquid phase. An NaOH trap to quantify $^{14}CO_2$ was set up as previously described (24). At intervals, 1-ml was removed from the buffer medium and analyzed for the presence of atrazine and potential atrazine metabolites by HPLC as described below. Aliquots were also removed from the NaOH trap and analyzed for trapped radioactivity.

Atrazine degradation in crude cell extracts. Cells were grown in atrazine liquid medium, harvested by centrifugation at the end of logarithmic growth, washed twice with 10 mM sodium phosphate buffer (pH 7.2), and resuspended in the same buffer at a concentration equivalent to an OD_{600} of 5.0. Crude cell extracts were prepared by passing washed cells three times through a French press operating at 12,000 lb/in^2. The lysate was centrifuged at 30,000 × *g* for 30 min at 4°C, and the supernatant was passed through a cellulose acetate filter with a pore size of 0.2 μm and immediately frozen at −70°C. The protein content of the resulting crude extract was assayed by a bicinchoninic acid assay and adjusted to yield a crude extract containing 1 to 3 mg of protein per ml. Atrazine was incubated with protein extracts for the times indicated in Results and extracted with ethyl acetate (1:1, vol/vol), and the radioactivity in both phases was counted in a scintillation counter.

Soil experiments. Soil from a 10-year-old herbicide spill site in Madison, Minn., containing 4% (wt/wt) aged atrazine, was diluted with atrazine-free soil of the same type from a neighboring field to yield a final atrazine concentration of 1,500 ppm. The soil was air dried, mechanically crushed to yield granules of 1 to 3 mm, and passed through a sieve with a pore size of 2 mm. All treatments were duplicates in 20-liter cyclindrical polypropylene containers, each containing 10 liters of soil (15 kg). A *Pseudomonas* sp. strain ADP culture, grown on atrazine medium, was harvested in the late exponential phase, washed, and resuspended in deionized water to yield an OD_{600} of 1.0. Soil treated with *Pseudomonas* sp. strain ADP was inoculated with 3 liters of cell suspension, amended with 750 ml of water, and thoroughly stirred. Treatments receiving sodium citrate were amended with 750 ml of water containing 30 g of sodium citrate. Treatments receiving only sodium citrate were amended also with 3 liters of deionized water. Soil in the control treatment was irrigated with 3.75 liters of deionized water. After 3 weeks of static incubation at 30°C, three 10-g soil samples were removed from each container and extracted overnight with 30 ml of 1:1 (vol/vol) ethyl acetate-hexane. The extract was analyzed by gas chromatography as described below.

Analytical methods. Gas chromatography was performed with a model 5890 gas chromatograph (Hewlett-Packard, Palo Alto, Calif.) equipped with an autosampler, a nitrogen-phosphorus detector, and a cross-linked 5% phenylmethylsilicone capillary column (30 m by 0.2 mm [inner diameter]; J. T. Baker, Phillisburg, N.J.). The operating conditions were as follows: injector temperature, 240°C; detector temperature, 250°C; oven temperature, 160 to 220°C at 20°C/min; carrier flow rate, 1.5 ml/min. HPLC was performed with a model 8800 pumping system (Spectra Physics, San Jose, Calif.) equipped with a model 7125 valve-and-loop injector (Rheodyne, Cotati, Calif.), fitted with a 100-μl loop, and a model UVIS-204 detector (Linear Instruments, Inc., Reno, Nev.) operating at an absorbance wavelength of 220 nm. The chromatography protocol of Vermeulen et al. (37) was followed, except that the isocratic mobile phase contained methanol-water (50:50, vol/vol) and 50 mM ammonium acetate. The flow rate was 1 ml/min, and the column was operated at room temperature. The chromatograms were analyzed on a Chromjet recording integrator (Spectra Physics). Authentic samples were cochromatographed to aid in the identification of the metabolites.

RESULTS

Isolation of a single bacterium metabolizing atrazine as sole nitrogen source. In a previous study (24), atrazine was mineralized by stable mixed cultures obtained from soils exposed to herbicide spills, but a pure culture was not obtained. However, repeated subculturing resulted in increasing rates of atrazine degradation. In the present study, the use of advanced subcultures and atrazine indicator plates allowed a pure culture to be obtained. The indicator plate, containing atrazine as the sole nitrogen source at concentrations as high as 1,000 ppm, showed clearing zones around the bacterial growth (Fig. 1). This technique also indicated the ability of the bacterium to metabolize very high concentrations of atrazine in a solid matrix. Agar removed from clearing zones and placed on top of atrazine-containing agar did not exhibit atrazine-degrading ability. Therefore, it was assumed that extracellular enzymes do not play an important role in the degradation of atrazine. The purity of the culture was ensured by subculturing onto rich medium and then restreaking isolated colonies onto atrazine plates.

Taxonomy of the atrazine-metabolizing bacterium. The isolated bacterium was a gram-negative motile rod. It contained a single polar flagellum observable by electron microscopy. Bacterial colonies on atrazine solid medium were generally circular with an irregular border and were opaque and light brown with darker coloration in the center. Growth on King's medium B was very rapid, with the formation of fluorescent colonies in <24 h. The bacterium grew well at 42°C. It used glucose as a carbon source but did not use fructose, sucrose, galactose, lactose, or maltose. It was catalase positive and oxidase positive, it reduced nitrate, and it grew nonfermentatively on glucose as indicated on triple sugar iron agar. These observations suggested that the organism might belong to the RNA group I of the genus *Pseudomonas* (31).

Further tests were conducted that confirmed the isolate was a *Pseudomonas* sp. The results of tests involving the Biolog identification system and 16S rRNA sequencing are summa-

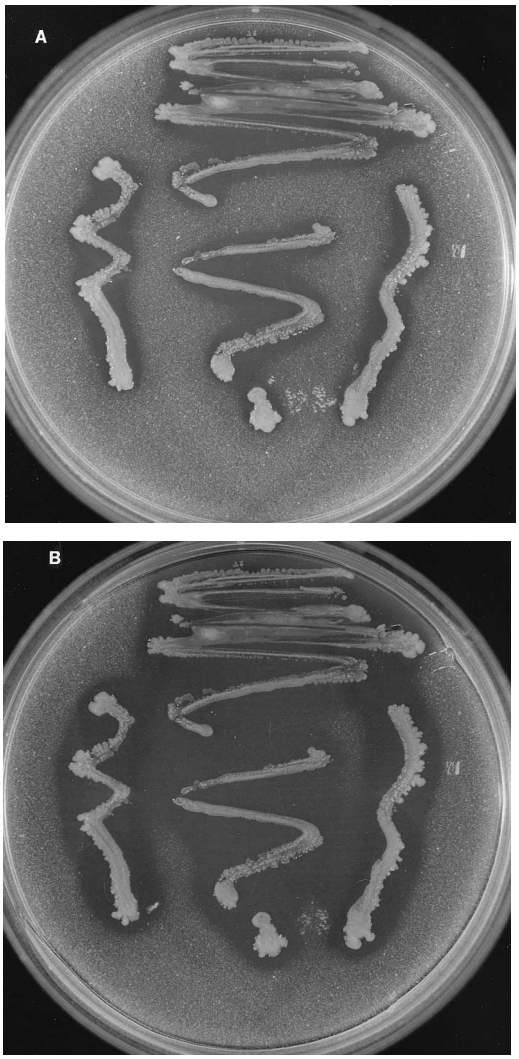

FIG. 1. Agar plates of the atrazine-degrading isolate showing clearing zones from the degradation of 1,000 ppm of atrazine. (A) 48 h after inoculation; (B) 72 h after inoculation. The atrazine agar medium is described in Materials and Methods.

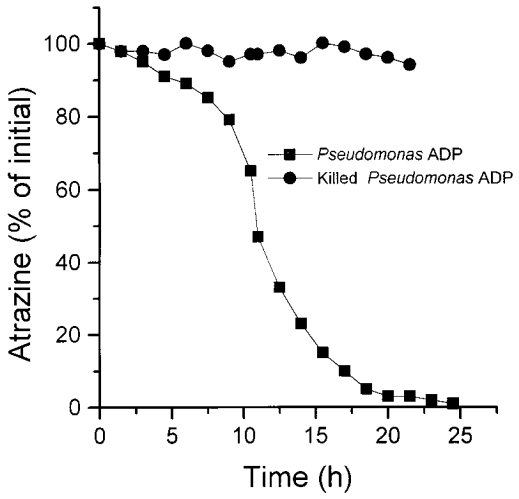

FIG. 2. Degradation of 100 ppm of [^{14}C]atrazine in a liquid medium by a growing culture of *Pseudomonas* sp. strain ADP. The control was inoculated with cells that had been boiled for 10 min.

rized in Table 1. The isolate was sufficiently similar to *P. citronellosis* in the Biolog biochemical tests to be considered a member of that species, and the 16S rRNA sequences showed 96.1% identity. However, preliminary fatty acid profiles obtained for the isolate indicated that the strain was an acceptable match with *P. aeruginosa*. Thus, the isolate is denoted here as *Pseudomonas* sp. strain ADP, which is not a precise match to any known species but most closely related to *P. citronellosis* in rRNA group I.

TABLE 1. Taxonomic comparison of atrazine-metabolizing isolate and type strains of *Pseudomonas* and *E. coli*

Comparison with:	Similarity index in Biolog test[a]	% Identity of 16S RNAs
P. citronellosis	0.554	96.1
P. aeruginosa	0.001	94.4
P. nitroreducens	0.007	—[b]
P. cepacia	<0.001	82.7
P. testosteroni	<0.001	80.8
E. coli	<0.001	82.8

[a] >0.50 is considered an acceptable match.
[b] Data not available for comparison.

Atrazine metabolism by growing cells. An aliquot from an atrazine-grown culture was transferred into sterile liquid atrazine medium containing [^{14}C]atrazine, and immediate growth was observed. After 24 h, 0.46 mM [^{14}C]atrazine (100 ppm) was completely metabolized, as evidenced by HPLC analysis of the culture medium (Fig. 2). A control with heat-killed bacteria did not show a significant decrease in atrazine concentration. Bacterial growth, as indicated by an increase in OD_{600}, was observed to occur concomitantly with atrazine disappearance. To preclude artifacts, the protein content of cultures was also determined as a function of time. Figure 3 shows that the protein concentration increased similarly with cells grown on atrazine and on ammonium nitrate. In separate ex-

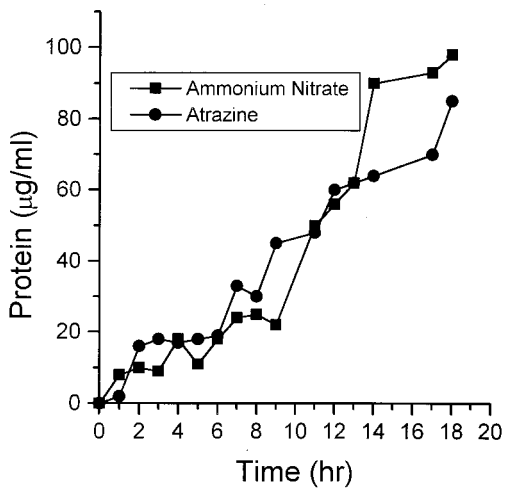

FIG. 3. Increase in protein content in a culture inoculated with *Pseudomonas* sp. strain ADP and containing atrazine or ammonium nitrate as the sole nitrogen source.

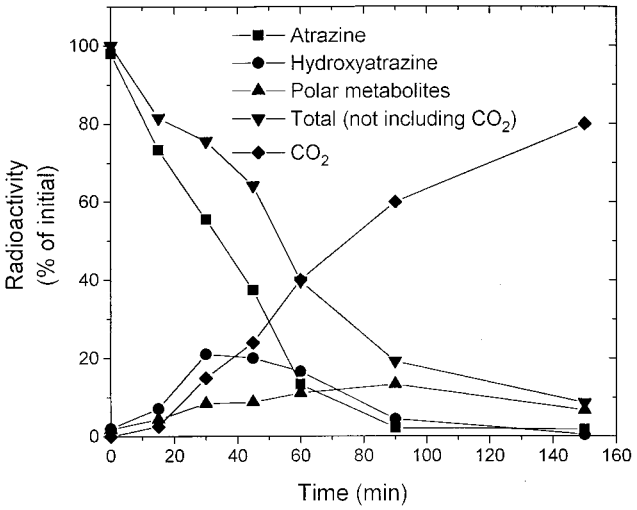

FIG. 4. Metabolism of 100 ppm of [^{14}C]atrazine by resting-cell suspensions of *Pseudomonas* sp. strain ADP showing formation of water-soluble metabolites and, ultimately, CO_2.

periments, viable bacterial counts were also found to increase concomitantly with atrazine disappearance (data not shown).

Atrazine degradation by nongrowing cultures. It was important to ascertain if *Pseudomonas* sp. strain ADP could metabolize atrazine under nongrowth conditions. Cells grown on atrazine were harvested in the late exponential phase, washed, and resuspended in fresh medium to a density of 1.15×10^{10} CFU per ml (1.5 mg of protein per ml). Under these conditions, 0.46 mM atrazine (100 ppm) was completely metabolized in less than 150 min (Fig. 4). Ring-labeled [^{14}C]atrazine was used to indicate the completeness of metabolism. Eighty percent of the initial radioactivity was released and trapped as $^{14}CO_2$ (Fig. 4). While atrazine was metabolized immediately, stoichiometric quantities of CO_2 were not formed immediately. An analysis of the culture medium by HPLC revealed the presence of ^{14}C-compounds more polar than atrazine. A chromatograph of the culture medium after a 30-min incubation of atrazine with cells is shown (Fig. 5B), along with standard compounds (Fig. 5A) and a control incubation with heat-killed cells (Fig. 5C) for comparison. The major metabolite at the earlier time points comigrated with hydroxyatrazine. To confirm the identity of the product, the metabolite was purified and subjected to mass spectrometry. The mass spectrum was identical to that of authentic hydroxyatrazine (inset, Fig. 5B). Additionally, a more-polar metabolite(s) was observed to elute approximately 3 min after injection. Given the similar retention times of a number of potential atrazine metabolites, identification of additional metabolites will require further work. The time course for the formation of hydroxyatrazine and other polar compounds is shown in Fig. 4 and suggests that hydroxyatrazine is an intermediate in atrazine metabolism by *Pseudomonas* sp. strain ADP. Separate studies were performed with resting-cell suspensions that were not aerated. The metabolism of atrazine under these conditions was similar.

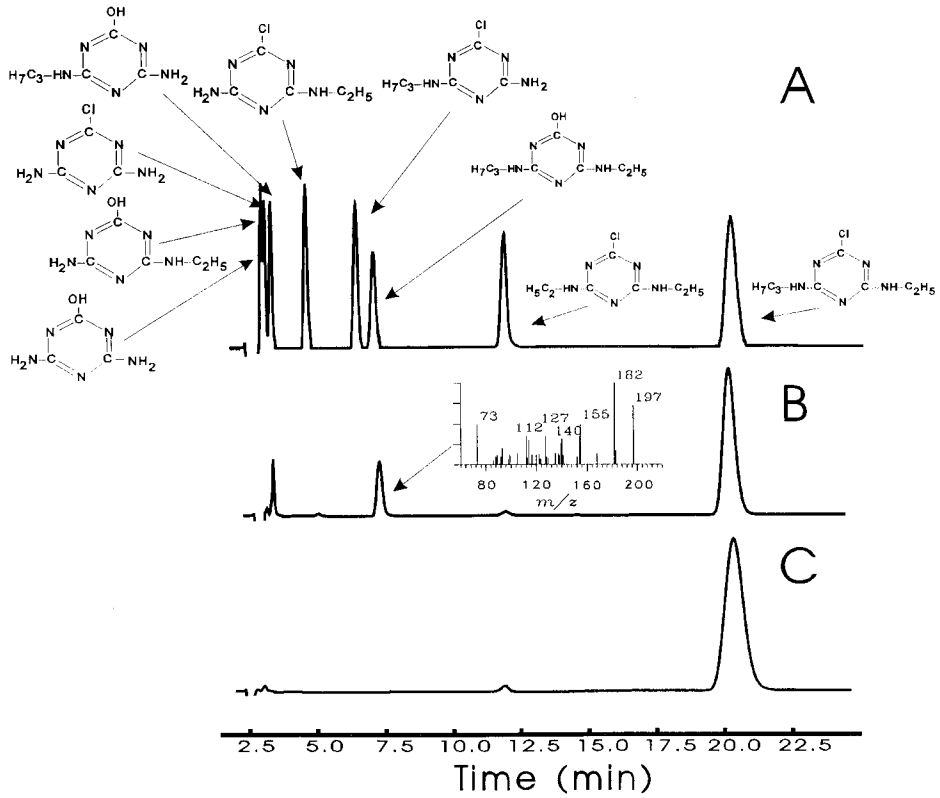

FIG. 5. HPLC of extracts from cell supernatants after metabolism of 100 ppm of atrazine by 9.2×10^9 resting cells per ml in 100 mM phosphate buffer (pH 7.2). (A) A reference HPLC chromatogram of atrazine, simazine, and possible atrazine metabolites; (B) HPLC chromatogram of the extracted incubation mixture after 30 min (the inset shows the mass spectrum of the material eluting at 7.5 min); (C) control chromatogram of an extract of atrazine in phosphate buffer prior to inoculation with cells.

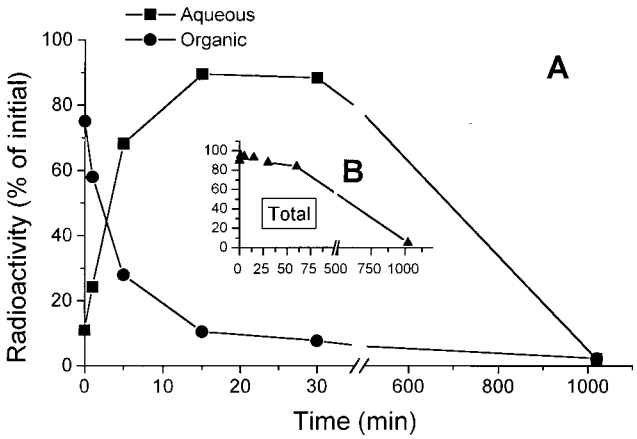

FIG. 6. [^{14}C]atrazine metabolism by cell extracts prepared from *Pseudomonas* sp. strain ADP. The initial atrazine concentration was 100 ppm. The concentrations of atrazine and water-soluble metabolites were determined as described in Materials and Methods. (A) Time course of the disappearance of organic-soluble atrazine and appearance and disappearance of water-soluble intermediates; (B) disappearance of total ^{14}C from the incubation mixture.

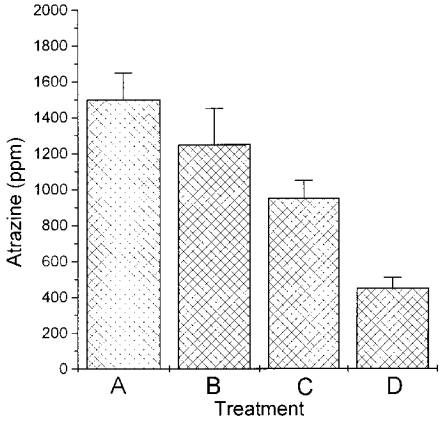

FIG. 7. Atrazine degradation in aged soil. The treatments were control, brought to 85% of water-holding capacity (A), *Pseudomonas* sp. strain ADP added (B), soil brought to 85% of water-holding capacity with water containing sodium citrate (C), and a combination of treatments B and C (D). The experimental details are described in Materials and Methods.

Atrazine degradation by cell extracts. Cell extracts were prepared as described in Materials and Methods. Cell-free protein supernatants prepared following centrifugation at 10,000 × *g* or 100,000 × *g* for >1 h were incubated with [^{14}C]atrazine. Both fractions transformed 90% of the atrazine to water-soluble materials in 1 h. Boiled cell fractions failed to metabolize atrazine. These data suggested that atrazine was transformed by soluble enzymes from *Pseudomonas* sp. strain ADP.

In another experiment, [^{14}C]atrazine was incubated with 1.5 mg of soluble protein per ml for various times, the reactions were quenched with organic solvent, and the two fractions were analyzed as described in Materials and Methods. The kinetic course of the metabolism is shown in Fig. 6. Within 15 min, most of the atrazine, extractable into organic solvent, was transformed into water-soluble materials. Over the course of hours, the radioactivity in the soluble fraction was depleted (Fig. 6, inset B), presumably going to CO_2 that volatilized out of the reaction mixture (Fig. 4). Boiled cell extracts did not show any loss of [^{14}C]atrazine from the organic solvent-extractable fraction of control reaction mixtures. The crude extract could be stored at −70°C for several months with no loss of activity.

Atrazine degradation in soil. Soil that had high levels of aged atrazine, resulting from a contamination occurring about 10 years before sampling, was obtained. Containers with 10 liters of soil were subjected to the following treatments: (i) addition of water only (control), (B) addition of 2 × 10^6 *Pseudomonas* sp. strain ADP cells per g of air-dried soil, (iii) addition of 2 mg of sodium citrate per g of air-dried soil, and (iv) addition of a mixture of *Pseudomonas* sp. strain ADP and sodium citrate at the concentrations used in treatments ii and iii. Sodium citrate amendment was used because it had previously been shown to significantly stimulate atrazine biodegradation by *Pseudomonas* sp. strain ADP in small-scale laboratory experiments (23). The incubation was conducted for 3 weeks, after which the soil was extracted and analyzed for residual atrazine by gas chromatography.

The results of this experiment are shown in Fig. 7. With the addition of water only, the concentration of atrazine in the soil remained constant at 1,500 ppm. With the addition of *Pseudomonas* sp. strain ADP alone, the concentration of atrazine declined to 1,250 ppm. With the addition of sodium citrate alone, the concentration of atrazine decreased to 975 ppm. The best effect was observed with the addition of a mixture of *Pseudomonas* sp. strain ADP and the stimulatory carbon source. Under these conditions, the residual atrazine concentration was 450 ppm, a decline of 70% from the initial concentration.

DISCUSSION

Atrazine is the most prevalent of the *s*-triazine formulations applied in the environment and is also less readily metabolized than certain other *s*-triazine compounds. A number of pure cultures have been found to metabolize cyanuric acid and ammeline as their sole source of nitrogen (8) but were unable to use atrazine. A recent report indicates that several different bacteria are capable of metabolizing atrazine, but few data were presented for pure cultures in that study (26). In the last few months, bacterial isolates have been found to use atrazine as a carbon source (38) and as a nitrogen source (33). In contrast to those previous studies, *Pseudomonas* sp. strain ADP metabolized atrazine completely at concentrations far exceeding the solubility of atrazine in water. Furthermore, the present study demonstrated that in vitro enzyme activity against atrazine can be obtained.

It is of interest that *Pseudomonas* sp. strain ADP was isolated from a stable mixed culture from which individual atrazine-metabolizing bacteria could not be previously obtained (24). This might reflect an increase in the numbers of this specific organism on repeated subculturing, the usefulness of the atrazine indicator plates, or the possibility of interspecies gene transfer generating a single competent strain. With respect to the last possibility, current studies on the molecular biology of atrazine metabolism may help to resolve that issue. The isolated bacterium did not precisely resemble any currently defined species. This is not an uncommon finding, given the diversity of soil bacteria and the likelihood that only a small fraction have been characterized to date. However, its rigorous identification here as a *Pseudomonas* sp. in rRNA group I is important for developing molecular biological approaches for the study of bacterial atrazine metabolism.

Pseudomonas sp. strain ADP metabolized atrazine as its sole nitrogen source during growth. Atrazine nitrogen was clearly

being assimilated, as evidenced by the increase in protein concentration concomitant with atrazine metabolism. Furthermore, the atrazine ring was shown to be cleaved and the carbon atoms to be liberated as CO_2. *Pseudomonas* sp. strain ADP also metabolized atrazine under nongrowth conditions. This is significant in the context of potential environmental applications of the organism, for which amended or immobilized cells might be used for the bioremediation of atrazine. The data indicate that *Pseudomonas* sp. strain ADP will continue to metabolize atrazine after its nitrogen needs have been met. At least some of the enzymes responsible for atrazine metabolism are found in the cell cytoplasm or periplasm, as evidenced by the localization of enzyme activity in the cell extract supernatant fluid following centrifugation at 100,000 $\times$ *g*. The apparent stability of cell-free activity suggests that further enzymological studies of atrazine metabolism are possible with this organism as a model system.

Experiments with aged soils containing high levels of atrazine demonstrated that several treatments were effective in degrading atrazine. The addition of *Pseudomonas* strain ADP was somewhat effective (Fig. 7), indicating that this organism can survive and metabolize atrazine in a soil environment. In this context, it is important to further investigate the survivability and metabolic persistence of *Pseudomonas* sp. strain ADP in soils. The most important factor stimulating atrazine biodegradation was the addition of sodium citrate (Fig. 7). In laboratory studies, sodium citrate was an excellent carbon source for supporting atrazine metabolism, and this knowledge was applied effectively to remove atrazine from soil. The removal of more than 1,000 ppm of atrazine by a combination of *Pseudomonas* sp. strain ADP and sodium citrate was impressive, particularly because the herbicide was spilled 10 years previously and aged soils are, in many cases, difficult to treat biologically. The effectiveness of sodium citrate alone suggests that indigenous bacteria, perhaps resembling *Pseudomonas* sp. strain ADP, are present and require only the proper conditions to stimulate the metabolism of atrazine. The results obtained with the control, amended with water to levels comparable to the other treatments, ruled out the possibility that soil moisture content alone was important for stimulating atrazine degradation.

The studies with liquid cultures indicate the potential for *Pseudomonas* sp. strain ADP to completely metabolize the *s*-triazine ring of atrazine to CO_2, but this remains to be established for cultures in soil. It is important to note that we detected hydroxyatrazine as a transient intermediate in both culture filtrates of whole cells and cell extract incubations and that this compound has been widely detected in atrazine-contaminated soils (1, 14, 28). Hydroxyatrazine is more strongly bound to soil organic matter than is atrazine and may be sequestered from further metabolism in soil under some conditions. Our findings here support the idea that hydroxyatrazine in soil may be derived largely from biological hydrolysis reactions. We previously showed that a mixed microbial culture incorporated ^{18}O from $H_2{}^{18}O$ into atrazine, yielding [^{18}O]hydroxyatrazine (25). The studies here with the pure culture are consistent with those previous findings. Prior to these reports, hydroxyatrazine formation in the environment was proposed to result solely from chemical hydrolysis (1, 6, 14, 18, 20, 28). The dechlorination of atrazine removes its herbicidal properties and is proposed to decrease its toxicity (21). Further studies will focus on atrazine metabolites of *Pseudomonas* sp. strain ADP and genes and enzymes that mediate atrazine metabolism.

ACKNOWLEDGMENTS

We thank Janis McFarland of Ciba-Geigy Corporation for providing *s*-triazine standards. The assistance of Greg Brusseau and Richard Hanson with sequencing of bacterial 16S rRNA and of Tom Krick with mass spectrometry is gratefully acknowledged. We thank Bonnie Allen for assistance in the preparation of the manuscript, David Dix for technical assistance, and Mervyn DeSouza for information and discussion on the taxonomy of the isolate.

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