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Degradation of Atrazine by *Pseudomonas*: N-Dealkylation and Dehalogenation of Atrazine and Its Metabolites[1]

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Three species of *Pseudomonas* capable of utilizing atrazine as a sole source of carbon were isolated by enrichment from soil with a long history of atrazine application. Atrazine was metabolized via N-dealkylation with preferential formation of deisopropylatrazine over deethylatrazine. Two of the species were able to carry out the dechlorination of both deisopropylatrazine and deethylatrazine following incubation in glucose-supplemented mineral salts medium. The dehalogenation of atrazine and its metabolites as a bacterial degradation process is shown.

INTRODUCTION

Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine] is a widely used selective herbicide for weed control in corn. It has been shown that the metabolism of atrazine in soil involves hydroxylation, dealkylation, and ring cleavage (Esser et al., 1975). Phytotoxicity of the herbicide is destroyed by hydroxylation at the 2-position but not by dealkylation of either of the two alkylamino groups (Kaufman and Blake, 1970). Dealkylation reactions in soil have been shown mainly due to fungi (Couch et al., 1965; Kaufman and Blake, 1970; Wolf and Martin, 1975). Giardina et al. (1979) isolated a *Nocardia* species from a soil enrichment culture that can utilize atrazine as the sole source of carbon and nitrogen. Their metabolic studies with this organism showed both N-dealkylation and deamination of atrazine and the formation of 2-chloro-4,6-diamino-*s*-triazine (Giardina et al., 1980, 1982), which was reported as nonphytotoxic in bioassays with oats.

Dechlorination of atrazine to a nonphytotoxic product, hydroxyatrazine, occurs in soils treated with atrazine, but this has been attributed solely to chemical hydrolysis rather than resulting from microbial activity (Armstrong et al., 1967; Skipper et al., 1967; Kaufman and Blake, 1970). According to Knackmuss (1981) the bacteria have not evolved enzymatic systems for the direct hydrolysis of the aromatic carbon–halogen bond. Klages et al. (1981), however, have reported such activity in whole cells.

Very little is reported in the literature on the metabolism of atrazine by *Pseudomonas*. This is a ubiquitous genus, genetically well characterized, and shown to have a wide range of catabolic pathways including the capability to degrade oil spills and a large number of other herbicides (Wheelis, 1975; Chakrabarty, 1976; Clark, 1982; Haas, 1983). Bryant (1963) isolated three groups of *Pseudomonas* species from enrichment cultures of soil treated with seven *s*-triazine compounds. However, only one of the species could grow on atrazine as the sole source of carbon. The abundance of this *Pseudomonas* species in his enrichment culture was dependent on the presence of an ethylamino group at the 4-position of the *s*-triazine ring. Cook and Hutter (1981) have isolated and described *Pseudomonas* species capable of utilizing various *s*-triazines as the sole source of nitrogen. However, atrazine was not employed in their studies.

This study is a part of our research program involving the isolation of bacteria from the soil capable of degrading certain pesticides with a view to determine the intracellular localization of genes responsible for such biodegradation. The investigation reported in this paper describes the isolation of bacteria, belonging to the genus *Pseudomonas*,

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that utilize atrazine as a sole source of carbon for growth and degrade the herbicide to the hydroxy and dealkylated metabolites.

MATERIALS AND METHODS

Enrichment Cultures and Bacterial Isolation. Soil samples used as inoculum for enrichment isolation were taken randomly from four different areas of a corn field treated with commercial formulation of atrazine every year for the past 14 years. Atrazine application preceded the soil sampling for this study by 9 weeks. The enrichment medium, basal minimal salts nitrogen (BMN), contained (g/L) the following: K_2HPO_4, 1.6; KH_2PO_4, 0.4; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2$, 0.025; NH_4NO_3, 0.5. The pH was adjusted to 6.8–7.0. This was supplemented with 50 ppm of analytical grade atrazine (99% pure) and the medium designated as BMNA. Enrichment cultures were obtained simultaneously by the dilution method and by the soil percolation technique. In the dilution method, 2.5 g of soil samples were incubated with 40 mL of sterile BMNA medium with shaking at 28 °C with and without the addition of cycloheximide (0.1%) to inhibit the growth of fungi. Dilutions (0.5 mL into 25 mL of fresh medium of the same composition) were made every 2 weeks for 2 months. Finally 0.1 mL was added to 10 mL of BMNA medium, the resultant mixtue was incubated for 10 days, and the dilutions were plated on BMNA agar (1.5% Difco agar) plates. Fast-growing single colonies of bacteria (about 10–12) were picked from under a dissecting microscope (generally after 7–10 days) and purified by streaking on BMNA–agar plates and finally on BMNA–agarose (1.2% agarose) plates. Four passages on agar plates were sufficient to obtain purified uniform single bacterial colonies.

To obtain enrichment cultures by the soil percolation technique, 3 g of soil was mixed with 3 g of sterile fine sand and 2 g of glass beads and the mixture was placed in the percolation tube over a bed of glass wool. The percolation medium (100 mL) for culture enrichment contained, in addition to BMNA, 0.09% cycloheximide, 0.014% 2,3,5-triphenyltetrazolium chloride (TTC), 9 μg/mL basic fuschin, 10 μg/mL nitrofurantoin, and 20 μg/mL nalidixic acid (designated BMNA-GH or selective medium). The final pH of the medium was 6.8. The addition of the compounds to the enrichment medium was designed to inhibit the growth and multiplication of most of other microbial species except soil *Pseudomonas* as reported by Grant and Holt (1977). The soil percolation was carried out at room temperature (24–25 °C) for 5 weeks when the reservoir medium became slightly turbid. An aliquot was diluted (1:10) with fresh sterile BMNA–GH medium in a flask and shaken at 25 °C for 3 weeks. The dilutions were plated on BMNA–GH agar. Two types of colonies (large and small), which appeared after 7 days of incubation, were purified by successive transferring 3–4 times onto BMNA agar. Colonies were selected and replicated on the following media for preliminary characterization: TY (tryptone 5%, yeast extract 3%), Grant and Holt (1977) medium, King's A and King's B (Stolp and Gadkarni, 1981), and *Pseudomonas* isolation agar (Difco). Eight of the ten purified colonies appeared to belong to the *Pseudomonas* group. They were characterized according to *Bergey's Manual of Determinative Bacteriology* (1974) and further characterized according to the dichotomous key for the identification of the *Pseudomonas* species as described by Stolp and Gadkarni (1981). The purified colonies grown in TYC (TY containing 0.06% $CaCl_2$) medium with 50 μg/mL atrazine were stored at −70 °C with 40% glycerol (final concentration).

Assays of Atrazine and Its Metabolites. Atrazine degradation in incubated soil samples (flask and percolator) was determined by the decrease in its concentration. Utilization of atrazine as a carbon source by the isolates was determined by the increase in protein content of the cultures with two concentrations of atrazine. This was further verified by the increase in viable cell count determinations.

Table I. Atrazine Degradation in Soil-Enrichment Cultures[a]

enrichment medium	atrazine, ppm initial	atrazine, ppm after 5 weeks	% degradn
uninoculated	51.7	50.8	
BMNA	53.4	29.6	44.5
BMNA–GH[b]	49.5	41.2	16.7
BMNA + cycloheximide	51.8	35.9	30.6

[a] Average of two samples. [b] Incubated in the presence of inhibitors described by Grant and Holt (1977) for the isolation of *Pseudomonas* species.

Biodegradation and Utilization of Atrazine by the Cells. Cells grown overnight on Ty–agar plate were suspended in BMN medium. They were shaken for 2 h at 28 °C to deplete the carry-over nutrients. Aliquots of the suspension (1 mL each) were added to 3 mL of BMN and BMNA medium (atrazine concentration 60 μg/mL) and the cultures incubated with continuous shaking for 2 weeks. The number of viable cells in the cultures was determined before and after the incubation period. Aliquots in duplicate were taken, and dilutions were made with BMN as the diluent. Appropriate dilutions in triplicate were spread on nutrient agar plates. The average of the number of colonies on the three plates was used to calculate the cell number.

Dehalogenation of Deisopropylatrazine, Deethylatrazine, and 2-Chloro-4,6-diamino-*s*-triazine. The dechlorination of the atrazine metabolites by the *Pseudomonas* species was examined by incubating the compounds in BMN medium supplemented with 3 mg/mL glucose for 5 weeks. The extent of dehalogenation was determined in the samples by the formation of hydroxy analogues.

Analysis of Atrazine and Its Metabolites. An aliquot of the medium (0.5 mL) was taken in a round-bottom flask and evaporated just to dryness at room temperature with a gentle stream of dry nitrogen. The material was dissolved in methanol, and an aliquot was analyzed directly by GC while another portion was methylated with diazomethane prior to GC analysis.

Gas Chromatography (GC). The gas chromatograph was a Varian Model 6000 fitted with a thermionic specific detector. The column was a 1.8 m × 0.26 cm i.d. glass tube packed with 3% Carbowax 20 M coated on 100–120-mesh Supelcoport. The operating conditions were as follows: column, detector, and injector port temperatures were 190, 300, and 220 °C, respectively; the nitrogen carrier gas, hydrogen, and air flow rates were 20, 4, and 150 mL/min, respectively.

Confirmation. The identity of the compound was confirmed by comparing the GC retention times with those of authentic samples, by cochromatography, and finally by gas chromatography–mass spectrometry. A high-resolution mass spectrometer, Model VG2AB-2F, connected to a Varian GC Model 3700 was used. The mass spectra were recorded at 70 eV.

RESULTS

Degradation of Atrazine in Soil-Enrichment Culture. Preliminary experiments showed that the herbicide was not degraded by any measurable extent after incu-

Table II. Antibiotic Resistance of *Pseudomonas* Isolates from Atrazine Enrichment Cultures

strain	antibiotics[a]				
	$Carb_{200}$	Cm_{200}	Gm_{100}	Kan_{100}	TC_{50}
189	+	+	-	-	-
194	+	+	+	+	+
S55	+	-	-	-	+

[a] Key: Carb, carbenicillin; Cm, chrloramphenicol; Gm, gentamycin, Kan, kanamycin; Tc, tetracyclin. Subscripts refer to the concentrations (μg/mL).

bation for 5 weeks with the soil samples taken from a field never exposed to atrazine. However, degradation was observed when the herbicide was incubated with the soil from a corn field that had a prolonged history of annual atrazine applications. Table I shows the amount of atrazine degraded under various conditions of incubation over a period of 5 weeks with the soil. The degradation of atrazine during enrichment with treated soil was considerably greater in BMNA medium (45%) than BMNA–GH (*Pseudomonas* predominant) medium (17%). However, addition of cycloheximide in the BMNA medium decreased the degradation of atrazine only moderately (from 45 to 31%). It was observed that a 5-week incubation of atrazine in the soil enrichment media resulted in the formation of the two mono-N-dealkylated products (Table I).

Characterization of *Pseudomonas* Species in Enrichment Cultures. The results (Table I) of atrazine degradation in soil-enrichment cultures with BMNA–GH medium indicated that *Pseudomonas* may contribute considerably to the degradation of atrazine. Three groups of *Pseudomonas* species were isolated. On the basis of morphological and biochemical tests for taxonomic identification (Stolp and Gadkarni, 1981), these species were tentatively assigned to *Pseudomonas putida* (four isolates, 189, 192, 193, 195), *Pseudomonas fluorescens* (one isolate, 194), and *Pseudomonas stutzeri* (one isolate, S55). The isolates of the three species showed differential sensitivity to various antibiotics (Table II).

Growth of *Pseudomonas* Isolates with Atrazine as Sole Carbon Source. *Pseudomona* isolates, as shown by colony formation on BMNA agar plates vs. no growth on BMN agar, indicated utilization of atrazine as a sole source of carbon and energy. The growth of the cultures following single-colony inoculation into BMNA liquid medium was slow, indicating the necessity for the bacterial population to build up before significant amounts of utilizable metabolite(s) were formed. The utilization of atrazine as carbon source for growth by the isolates was ascertained, therefore, by incubating pregrown cells in BMNA medium and determining the increase in cell number after 2 weeks. The data in Table III show the utilization of atrazine as carbon source for growth of the bacteria. In a similar experiment cells of the isolates grown on BMNA–agar plates were suspended in BMNA medium containing 20 or 40 μg/mL atrazine, and the cultures were incubated with shaking for 2 weeks at 28 °C. The protein content of the cultures increased from the initial concentration of 22 μg/mL (isolate 194) to 30 and 56 μg/mL with the low and high atrazine concentrations, respectively. Similar results were obtained with isolates 192 and S55.

N-Dealkylation of Atrazine by the *Pseudomonas* Isolates. N-dealkylation of atrazine by the isolates was followed in BMNA medium supplemented with 2 mg/mL glucose or 1 mg/mL glycerol. It was observed that the amounts of deisopropylated atrazine (4.2–7.2 μg/mL) were considerably higher than deethylatrazine (0.08–0.3 μg/mL) in the incubation mixture with the isolates after 16 days of incubation.

Table III. Growth of *Pseudomonas* 192, 194, and S55[a] on Atrazine as the Carbon Source

strain (medium)	viable cell/mL ($\times 10^7$)	
	initial	after 14 days
192 control (BMN)	1.72	3.95
192 (BMNA)	1.48	19.40
194 control (BMN)	2.64	5.84
195 (BMNA)	3.10	16.72
S55 control (BMN)	2.16	5.56
S55 (BMNA)	2.42	14.90

[a] Cells of isolates 192, 194, and S55 grown overnight on TY–agar plates were suspended in BMN and shaken for 2 h to deplete the nutrient carryover. A 1-mL sample of the suspension from each was then added to 3 mL of BMN and BMNA (60 μg/mL atrazine) liquid media. The number of viable cells in the cultures was determined after 14 days of incubation at 29 °C by plating the dilutions on nutrient agar.

Table IV. Degradation of Dealkylated Metabolites of Atrazine by *Pseudomonas* Isolates 192 and 194[a]

compd	hydroxy analogue, %	
	strain 192	strain 194
deethylatrazine	51.2	16.7
deisopropylatrazine	48.7	15.2
2-chloro-4,6-diamino-*s*-triazine	68.0	24.3

[a] The degradation was followed in BMN medium supplemented with 2 mg/mL glucose and 40 μg/mL dealkylated compound. The formation of hydroxy analogues was determined after 5 weeks.

Dehalogenation of Metabolites. Two strains, 192 and 194, were effective in carrying out the dechlorination of all three compounds tested (Table IV). It was observed that the former strain was considerably more effective than the latter. However, the third species (strain S55) was ineffective in dechlorinating the compounds. There was no dechlorination in the control (uninoculated) samples, thereby indicating that these compounds in BMN medium were stable.

DISCUSSION

Although biodegradation of atrazine in soil has been attributed mainly to fungi (Kaufman and Blake, 1970; Kaufman and Kearney, 1970; Wolf and Martin, 1975), the results obtained in this study (Table I) indicate that a considerable portion of atrazine (50–60%) incubated with soil previously exposed to the herbicide was apparently degraded by nonfungal microbial species (incubation in the presence of cyclohexamide). The *Pseudomonas* species isolated from the herbicide-treated soil contributed up to 25–30% biodegradation of atrazine in the BMNA–GH medium. This may reflect the adaptive or genetic evolution of microbial species in response to the continual application of the herbicide to soil. Microbial species able to degrade chemicals previously considered to be recalcitrant have been isolated (Cook et al., 1983), and the possibility of developing bacteria with multiple degradative capability by genetic manipulation has been the subject of considerable interest in recent years (Chakrabarty et al., 1984). The isolation of *Pseudomonas* species, by modified enrichment culture technique, from our soil was not surprising. The members of this genus are known to possess a wide range of biosynthetic and catabolic activities and degradative plasmids (Stanier et al., 1966; Wheelis, 1975; Chakrabarty, 1976; Franklin et al., 1981; Clark, 1982).

Pseudomonas species in this study caused N-dealkylation by removing either the isopropropyl or the ethyl moiety of atrazine. Furthermore, it was observed that the formation of deisopropylatrazine was considerably greater

than that of deethylatrazine. The reason for the preferential *N*-dealkylation of isopropyl moiety is not known. Previous studies on atrazine degradation by fungi (Kaufman and Blake, 1970; Wolf and Martin, 1975) have shown deethylatrazine as the main dealkylated metabolite.

In earlier studies, hydroxyatrazine formation in trace amounts in atrazine soil-enrichment cultures or during metabolic studies with fungi (Couch et al., 1965; Kaufman and Blake, 1970; Wolf and Martin, 1975; Giardina et al., 1979) was attributed to chemical hydrolysis (Armstrong et al., 1967; Skipper et al., 1967) rather than resulting from microbial activity. Dechlorination of atrazine was not observed in pure culture studies. The results in this study show (Table IV) that two *Pseudomonas* species, 192 and 194, carried out the dehalogenation when the cells were incubated with either of the mono-N-dealkylated product of atrazine. However, *Pseudomonas* isolate S55 did not show such dechlorination, thereby eliminating the possibility of chemical hydrolysis in the incubation medium. It is noteworthy that very little dechlorination of the parent compound, atrazine, was observed in the incubation system with species 192 and 194. Thus, it appears that the presence of both alkyl groups on atrazine may be inhibitory for bacterial dechlorination. However, it was observed that such dechlorination occurred after the elimination of one or both alkyl groups(s). The results in this study suggest that microbial dechlorination may occur in soil after the removal of one of the alkyl group of atrazine. Cook and Hutter (1984) have also reported dechlorination of deethylsimazine by *Rhodococcus corallinus*.

The approach used in this study to isolate predominantly *Pseudomonas* species in soil-enrichment cultures by incorporation of inhibitors for eliminating unwanted microbes could be applicable for other herbicides. It is essential, however, to ensure that the inhibitors are not utilized as carbon source by subsequent purification of isolates in inhibitor-free medium. In our early experiments, methanol employed as a solvent for atrazine in the enrichment studies was most effectively used by an isolated strain. However, the isolate grew very poorly on atrazine.

Involvement of naturally occurring plasmids in the biodegradation of pesticides has shown in a number of studies (Chakrabarty, 1976; Don and Pemberton, 1981; Pickup et al, 1983; Haas, 1983; Furukawa and Chakrabarty, 1982). Examination of *Pseudomonas* species 192, 194, and S55 for their plasmid content by Eckhardt (1978) gel electrophoresis using two strains of *P. putida* (Courtesy R. Wheatcroft) with known plasmid(s) as reference revealed no detectable plasmids. This may indicate that the strains carry either no plasmids, very big plasmid(s), or unstable plasmids. Alternatively, the atrazine degradative ability in these strains is encoded by the genes on the bacterial chromosomes.

Complete degradation of atrazine by bacteria to ammonia and carbon dioxide can now be envisaged. In our studies, the *Pseudomonas* species degraded atrazine preferentially to deisopropylatrazine, which has been shown by Cook and Hutter (1984) to be completely degradable by the combined action of the *Pseudomonas* and *Rhodococcus* isolates obtained in their studies.

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