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Degradation of Bromacil by a *Pseudomonas* sp.†

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A gram-negative rod, identified as a *Pseudomonas* sp., was isolated from soil by using bromacil as the sole source of carbon and energy. During growth on bromacil or 5-bromouracil, almost stoichiometric amounts of bromide were released. The bacterium was shown to harbor two plasmids approximately 60 and 100 kilobases in size. They appeared to be associated with the ability to utilize bromacil as a sole source of carbon and also with resistance to ampicillin. This microorganism also showed the potential to decontaminate soil samples fortified with bromacil under laboratory conditions.

Bromacil (5-bromo-3-sec-butyl-6-methyluracil) is generally used on noncropland areas for control of a wide range of annual and perennial grasses, broadleaf weeds, and certain woody species (13). It is also recommended for selective weed control in pineapple and citrus groves. Bromacil is considered to be moderately to highly mobile and relatively less biodegradable (14) than many other herbicides. It can persist in soil for at least 2 years in effective amounts (3) and has been shown to leach rapidly through soil toward groundwater, particularly during periods of heavy rainfall immediately after its field application (8). In addition, prolonged usage of bromacil has caused serious land-use problems in areas such as old citrus groves, where the cropping pattern may require change. In such cases, the land may remain unsuitable for other agricultural use for many years, until the bromacil residues descend to levels tolerable by the desired crop plants.

Degradation of this herbicide in soil by chemical oxidation (2) and photodecomposition (10) has been found to be negligible. Most degradation has been reported to be accomplished microbially (7, 17), and yet little is known about the soil microorganism(s) responsible for the degradation of bromacil. The biochemical pathways involved in the degradation of bromacil are not clear either. It is important to understand the metabolic pathways and resulting metabolites of xenobiotics released in large quantities to the environment. These compounds, which may not be toxic at the normal concentrations found in soils and waters, may be converted into more toxic forms (16) or may accumulate in the environment through the food chain (6).

In this report, we describe the isolation and properties of a *Pseudomonas* sp. capable of degrading bromacil. This bacterium was found to harbor two large plasmids. Preliminary evidence for involvement of the plasmid(s) in degradation of the herbicide is also provided.

MATERIALS AND METHODS

Chemicals. Analytical grade bromacil (purity, >99%) was obtained from the U.S. Environmental Protection Agency, Research Triangle Park, N.C., and from Chem Service, West Chester, Pa. Other organic chemicals were purchased from Fisher Scientific Co., Orlando, Fla., and from Sigma Chemical Co., St. Louis, Mo.

Medium and culture conditions. *Pseudomonas* sp. strain 50235 was maintained aerobically at 30°C on minimal medium (4) containing bromacil as the sole source of carbon.

Isolation and identification of *Pseudomonas* sp. strain 50235. To isolate bacteria capable of degrading bromacil, we followed a previously described enrichment procedure (4). A bacterial isolate which rapidly degraded bromacil was tentatively identified as a *Pseudomonas* sp. by the criteria of Stanier et al. (15). Ability of this isolate to utilize various other compounds as a sole source of carbon was also determined. To determine MICs, we added antibiotics and metal salts (as the sulfates or chlorides) to the growth medium as desired. Scanning electron microscopy was performed following a standard procedure (1).

To estimate the degradation of bromacil by the *Pseudomonas* sp., minimal medium containing the herbicide as the sole source of carbon was inoculated with the freshly grown cells and incubated at 30°C. Samples were withdrawn from the incubation mixture and centrifuged, and the supernatant and pellet were analyzed by spectrophotometrically or by high-performance liquid chromatography as described below. Bromide ion concentrations were determined with a bromide electrode (Fisher).

Degradation of bromacil in fortified soil samples. A separately sterilized sandy soil (pH 6.5) was placed into autoclaved glass jars (100 g per jar). All jars were then supplemented with constituents of the respective treatments (Table 1) and with water (one-third of field capacity). The contents of each jar were inoculated with the *Pseudomonas* sp., carefully mixed, and incubated at 28°C. Identically treated uninoculated control jars were maintained. At predetermined intervals, 10 g of soil was withdrawn from each jar for determination of residual bromacil by the method of Joliffe et al. (9), with the following modifications. Alkaline extraction of the soil samples was repeated three times, and pH of the aqueous alkaline extract was carefully adjusted. Extracted samples in acetone were adjusted to the appropriate volume for analysis by high-performance liquid chromatography with chloroform-water (55:45) as the solvent system. Sensitivity of the method was 2 ng per sample. Extracts in aqueous solution and culture supernatant were routinely analyzed spectrophotometrically by scanning from 200 to 350 nm. Bromacil has strong absorption maxima at 211 and 275 nm, and disappearance of the peak at 275 nm was regarded as evidence for degradation of the herbicide.

Oxygen uptake assay. Cells grown in 100 ml of LB (yeast extract, 5 g; tryptone, 10 g; NaCl, 10 g; in 1 liter of distilled water with the pH adjusted to 7.0) were harvested in the mid-exponential growth phase by centrifugation (10,000 × *g*

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TABLE 1. Growth of the *Pseudomonas* sp. in minimal medium containing various organic compounds as the sole source of carbon

Carbon source	Concn used (%)	Growth	Generation period (h)
Glucose	1	+	3
Fructose	1	−	
Sodium acetate	1	+	5
Sodium benzoate	1	+	6
Sodium gentisate	0.5	+	5
Sodium glutamate	1	+	3
Sodium malate	0.1	+	4
Sodium pyruvate	1	+	3
Sodium succinate	1	+	3
Catechol	0.1	−	
Sodium salicylate	0.1	+	10

for 10 min at 4°C), washed three times with minimal medium, suspended in the same medium (50 ml) containing an appropriate carbon source, and incubated overnight for induction of the degradative enzymes. The induced cells were then harvested, washed twice with phosphate buffer (0.1 M, pH 7.0), and suspended in the buffer to an A_{550} of 20. Oxygen uptake assays were done in a volume of 1.5 ml in a vessel equipped with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). The reaction mixture contained the phosphate buffer saturated with oxygen and cell suspension (9:1) to a final A_{550} of 2. After the background level of oxygen uptake was estimated, the reaction was started by the addition of a final concentration of 1 mM bromacil. For glucose, benzoate, gentisate, and 5-bromouracil, 2 mM (final concentration) solutions were used. A linear decrease in oxygen concentration was measured over 15 min. Controls were run simultaneously, using cell suspensions of the cured strain. Whole cells were suspended in 0.1 N NaOH containing 1% sodium dodecyl sulfate (SDS) before estimation of protein (11).

Enzyme assays. Crude cell extracts were prepared by sonicating the cells at 4°C with a Sonifier cell disrupter (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). They were centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant (crude cell extract) was then used for the 5-bromouracil reductase assay. The reaction mixture (1 ml) contained the phosphate buffer, 0.1 M KCl, 0.1 mM NADPH, and crude cell extract. After the background level of NADPH oxidation was estimated, the reaction was started by the addition of 1 mM bromacil. Reduction in A_{340} was used to estimate the reductase activity (the E_{340} of NADPH is 6,200). Protein was determined by the method of Lowry et al. (11), with bovine serum albumin as a standard.

Detection and isolation of plasmid DNA. An estimated 10^8 exponentially growing cells were suspended in 50 μl of E buffer (12) containing 25% sucrose, 2 mg of lysozyme per ml, and 100 μg of RNase per ml. The cell suspension was filled into the front wells of a 0.7% agarose gel in E buffer. A second series of wells immediately behind the front wells were filled with a lysis solution containing 1% SDS and 0.5% agarose in E buffer. Electrophoresis was first done for 30 min at 20 V for electrophoretic transfer of SDS into the front wells for cell lysis and then for 3 h at 100 V for fractionation of the DNA. To isolate plasmid DNA from the *Pseudomonas* sp., we suspended cell pellets from 1 liter of culture in 5 ml of 50 mM Tris hydrochloride (pH 8.0) containing 50 mM sucrose (TS) in a 250-ml centrifuge bottle. The suspended cells were treated with lysozyme (Sigma) by mixing with 5 ml of TS containing 5 mg of lysozyme per ml and 100 μg of RNase per ml, incubated at 60°C for 10 min, treated with 0.4 ml of 0.5 M EDTA added dropwise during gentle mixing, and incubated for another 5 min at room temperature. SDS (3 ml) (10%) and 2.3 ml of 1 N NaOH were then added and mixed by inversion, and the mixture was kept at room temperature until the suspension became a clear, viscous solution. This mixture was then neutralized by adding 3.7 ml of 2 M Tris hydrochloride pH 7.0. To remove the chromosomal DNA, we added 3 ml of SDS (10%) and 5 ml of cold 5 M NaCl separately to the mixture, which was then chilled in ice-water for 5 min, left at 4°C for 6 h, and centrifuged at 15,000 × *g* for 15 min. The supernatant was extracted with 5 ml of chloroform-phenol (saturated with 100 mM Tris hydrochloride pH 7.5) (1:1, vol/vol) and centrifuged, and the clear, aqueous top layer containing plasmid DNA was carefully removed into a fresh centrifuge tube. To this fraction, 45% (wt/vol) polyethylene glycol (3:1, vol/vol) was added, and the solution was mixed, chilled in ice-water for 5 min, and kept at 4°C for 6 h or overnight. Plasmid DNA was pelleted by centrifugation as above, washed with 70% ethanol, and dried in a Speed-Vac (Savant Instruments, Inc., Farmingdale, N.Y.). Dried DNA was suspended in TE buffer (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0) and purified by ethidium bromide-CsCl density gradient centrifugation (12).

Restriction enzyme cleavage analysis. Restriction endonucleases were obtained from U.S. Biochemical Corp., Cleveland, Ohio, and from International Biotechnologies, Inc., New Haven, Conn. Enzyme digestions were performed as specified by the manufacturers. Restriction enzyme-digested DNA was analyzed by electrophoresis through 0.8% agarose gels in E buffer (5, 12).

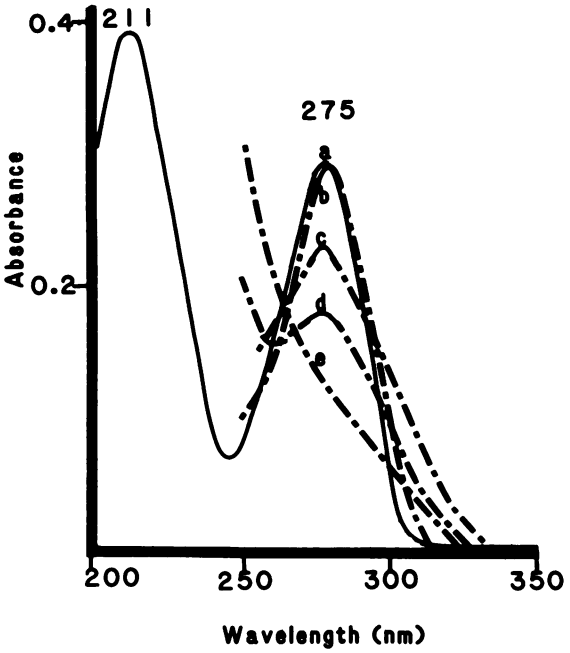

FIG. 1. Spectral analysis of the minimal medium containing bromacil incubated in the presence of the *Pseudomonas* sp. Shown are the minimal medium (containing 10 μg of bromacil per ml) (a) and the minimal medium after 0 h (b), 1 h (c), 2 h (d), and 3 h (e) of incubation with the *Pseudomonas* sp. (optical density at 550 nm = 1). The cells were removed by centrifugation before spectrophotometric analysis.

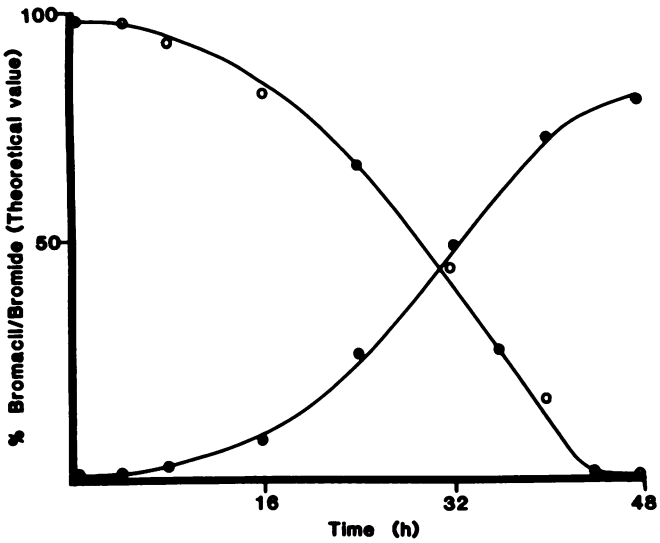

FIG. 2. Loss of bromacil (○) and appearance of bromide (●) in the culture supernatant of the *Pseudomonas* sp.

RESULTS AND DISCUSSION

Isolation and properties of *Pseudomonas* sp. strain 50235. Bromacil-contaminated soil samples were used to isolate pesticide-degrading microorganisms by an enrichment culture technique (4). Soil samples (5 g) were incubated aerobically in minimal medium (50 ml) in culture flasks at 28°C, with bromacil being used as the sole source of carbon. The incubation mixtures were subcultured once a week, and the bromacil concentration was gradually increased to 500 μg/ml. After 2 months, growth was evident in one of the flasks as judged by an increase in optical density as well as by disappearance of bromacil from the culture medium. This culture consistently degraded bromacil in subsequent subcultures. Plates of minimal medium containing bromacil were streaked from a subculture. Colonies that appeared during a 1-week incubation were patched and further streaked to isolate single colonies. One isolate, designated 50235, was able to grow on bromacil as demonstrated by bromide release and disappearance of the herbicide from the culture medium (Fig. 1 and 2) as well as by an increase in cell numbers. Strain 50235 was found to be a motile gram-negative rod approximately 1.0 by 0.3 nm in size as judged by scanning electron microscopy. It was oxidase, catalase, lipase, urease, and phosphatase positive. It hydrolyzed starch but not gelatin. None of the following carbohydrates was fermented by this bacterium: arabinose, cellobiose, fructose, galactose, lactose, maltose, mannose, rhamnose, or xylose. Strain 50235 was also screened with an API 20E test kit (Analytab Products, Plainview, N.Y.) and was identified as a *Pseudomonas* sp. It grew optimally at pH 7.0 and 30°C.

TABLE 3. 5-Bromouracil reductase activity in crude cell extracts of the *Pseudomonas* sp. grown on various carbon sources

Growth substrate	Sp act of 5-bromouracil reductase[a]
Glucose	0
Bromacil	215
5-Bromouracil	245
Benzoate	0

[a] In nanomoles per minute per milligram of protein.

Pseudomonas sp. strain 50235 was resistant to the heavy metals copper, cobalt, lead, and nickel (100 μg/ml) and mercury (50 μg/ml) and to the antibiotics ampicillin (100 μg/ml), chloramphenicol (5 μg/ml), lincomycin (40 μg/ml), and rifampin (10 μg/ml). It also utilized several other organic compounds as a sole source of carbon and energy including benzoate, gentisate, and salicylate as well as 5-bromouracil, a potential metabolite of bromacil degradative pathways (Table 1). It did not, however, grow on terbacil, 5-chlorouracil, catechol, or naphthalene. The doubling time of this organism on most of the substrates varied from 2 to 5 h for most substrates (i.e., 2 h on glucose, 3 h on malate and citrate, 5 h on acetate and bromacil).

Oxidation of organic compounds by whole cells. The metabolic pathway for the degradation of bromacil in *Pseudomonas* sp. strain 50235 was investigated by growing the organism on the substrates and monitoring the rate of oxygen uptake as affected by various possible intermediate products of metabolism (Table 2). Cells grown in minimal medium containing glucose did not utilize oxygen in the presence of bromacil. However, they were able to oxidize gentisate and benzoate. It was previously noticed that cells grown in LB require long lag periods for growth on bromacil and 5-bromouracil, suggesting that the enzymes involved in the degradation of these compounds were not produced constitutively. Bromacil-grown cells rapidly utilized oxygen in the presence of both bromacil and 5-bromouracil. However, 5-bromouracil-grown cells had low oxygen uptake when incubated with bromacil, indicating that enzymes involved in the initial steps of bromacil degradation may not be induced efficiently by 5-bromouracil.

5-Bromouracil reductase activity. The possibility that 5-bromouracil was an intermediate product was further investigated by measuring specific enzyme activities. The crude cell extracts catalyzed the oxidation of NADH or NADPH in the presence of 5-bromouracil, suggesting that the substrate was being reduced to 5-bromodihydrouracil. This activity was detected only in cells grown in minimal medium containing bromacil or 5-bromouracil as a sole source of car-

TABLE 2. Rates of oxygen uptake by washed cell suspensions of the *Pseudomonas* sp. grown on various carbon sources

Substrate	Rate of oxygen uptake (nmol of O_2 consumed min^{-1} mg of $protein^{-1}$) with the indicated carbon source for growth				
	Glucose	Benzoate	Bromacil	5-Bromouracil	Gentisate
Glucose	110	65	51	43	55
Benzoate	33	70	40	37	57
Bromacil	<2	<2	67	21	<2
5-Bromouracil	<2	10	65	60	<2
Gentisate	38	45	43	40	70
5-Chlorouracil	<2	<2	<2	<2	<2

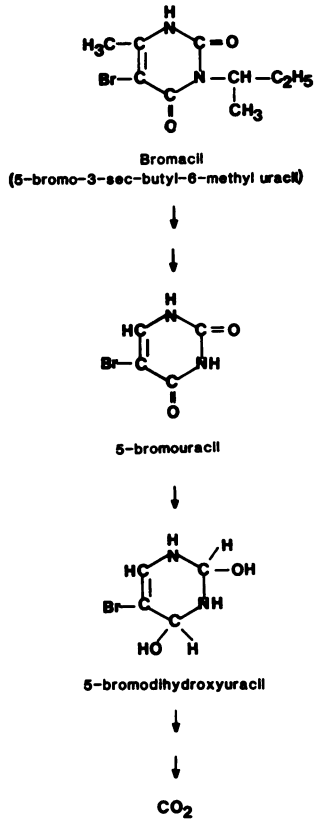

FIG. 3. Proposed pathways for the degradation of bromacil by the *Pseudomonas* sp.

bon (Table 3). It has been reported that the methyl and propyl moieties of bromacil are hydroxylated in soil to generate metabolites such as 5-bromo-3-sec-butyl-6-hydroxylmethyluracil, 5-bromo-3-(2-hydroxy-1-methylpropyl)-6-methyluracil, and 5-bromo-3-(3-hydroxy-1-methylpropyl)-6-methyluracil (7). No metabolite could be detected in the culture supernatants of the *Pseudomonas* sp. Based on the growth and reductase activities reported above, 5-bromouracil could be an intermediate of the metabolism of bromacil, and the metabolic pathway in the *Pseudomonas* sp. may be proposed as shown in Fig. 3. However, for elucidation of intermediate products further investigation is needed.

Degradation of bromacil in soil. To investigate the ability of the *Pseudomonas* sp. to degrade the herbicide in soil under laboratory conditions, we inoculated bromacil-fortified (50 μg/g) samples with the bacterium (10^5 cells per g). Most of the pesticide in samples inoculated with bacteria was degraded in 1 week, whereas no loss of bromacil was noticed in uninoculated samples over the same period. Doubling the amount of inoculum did not increase degradation of the pesticide. It was further found that the addition of other nutrients (nitrogen, potassium, and phosphorus at 0.5 μg/g) had little or no effect on the rate of bromacil degradation. However, the addition of 1 mg of glucose per g as a second carbon source almost completely stopped utilization of the pesticide by the microorganism. It is likely that the bacterium would start degrading bromacil in the soil after the easily metabolized alternative carbon source has been exhausted. Results in Table 2 support such a hypothesis because after 7 days of incubation of the fortified samples containing glucose, some loss of the herbicide was noticed. However, it was not as pronounced as in treatments with bromacil alone.

Bromacil residues adversely affect the productivity of some soils, and it is necessary to reclaim such soils to avoid economic losses. This can be accomplished by stimulating the activity of indigenous microorganisms responsible for the degradation of bromacil. However, all soils may not have indigenous bromacil-degrading microorganisms. Alternatively, isolated and cultured microorganisms such as the *Pseudomonas* sp. could be used to inoculate the affected soils to enhance degradation of the herbicide. Results of laboratory experiments with the *Pseudomonas* sp. suggest that the rate of degradation of bromacil in soil may be enhanced by inoculating soil with this microorganism. However, the efficacy of such inoculation under field conditions remains to be investigated.

Plasmids and bromacil degradation. Screening of strain 50235 for extrachromosomal DNA by a plasmid detection technique demonstrated the presence of two plasmids. Initial attempts to isolate these plasmids encountered a problem related to instability of the plasmid DNA, perhaps owing to nuclease activity which caused degradation of the plasmid DNA during its isolation. Incubation of the lysis mixture at 60°C instead of 37°C allowed the isolation of intact plasmid DNA (Fig. 4). The nature of the nuclease produced by this *Pseudomonas* sp. is currently being investigated. The isolated plasmids were approximately 60 and 100 kilobases in size. Restriction analysis of the combined DNA of both plasmids showed at least 22 and 25 fragments generated by endonucleases *Eco*RI and *Hin*dIII, respectively. Isolate 50235 lost its ability to degrade bromacil and its resistance to ampicillin (at a frequency of 10 and 2%, respectively) after 6

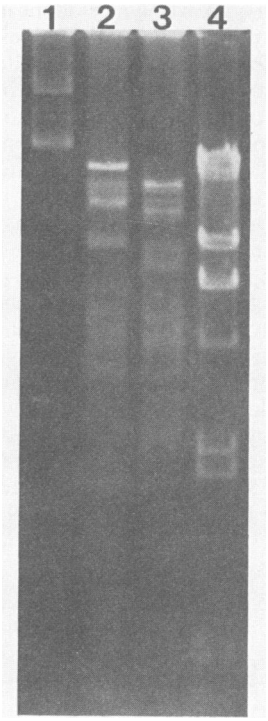

FIG. 4. Agarose gel electrophoresis of plasmid DNA isolated from the *Pseudomonas* sp. Shown are the plasmid DNA undigested (lane 1) and digested with *Eco*RI and *Hin*dIII (lanes 2 and 3, respectively). Molecular weight standards of lambda bacteriophage DNA cleaved with *Hin*dIII are in lane 4.

h growth in LB supplemented with mitomycin C (20 μg/ml) or streptomycin (50 μg/ml). However, other properties of the *Pseudomonas* sp. such as resistance to chloramphenicol, lincomycin, and rifampin were not affected. Both plasmids were not detected in strains lacking the ability to degrade bromacil (Brd^- strains) of the *Pseudomonas* sp., and there was no evidence for the occurrence of revertants (<1 in 10^9). These results suggest that one or both of the plasmids are involved in the degradation of the pesticide. Studies are under way to transfer these plasmids into *Pseudomonas putida*. It may then be possible to manipulate them and to study their structure and function in detail.

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