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Metabolism of twelve herbicides by *Streptomyces* *

D.R. Shelton, S. Khader, J.S. Karns[1] & B.M. Pogell[1]
USDA Agricultural Research Service, Natural Resources Institute, Environmental Chemistry Laboratory, Bldg 007, BARC-West, 10300 Baltimore Ave., Beltsville, MD 20705, USA; [1] *Soil Microbial Systems Laboratory*

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Abstract

Experiments were conducted to assess the ability of *Streptomyces* (strain PS1/5) to metabolize twelve herbicides representing several different classes including: acetanilides, triazines, ureas, uracils, and imidazoles. Incubations in aqueous culture with dextrin as carbon source and either ammonium or Casamino acids as nitrogen source resulted in transformations (> 50%) of eight of the herbicides tested: alachlor, metolachlor, atrazine, prometryne, ametryne, linuron, tebuthiuron, and bromacil; the remaining four herbicides (cyanazine, diuron, metribuzin, and imazapyr) were also transformed, but to a lesser extent. In most instances, biotransformations occurred concurrently with growth and results were consistent regardless of the nitrogen source (ammonium vs. Casamino acids). However, in some instances there were differences in rates of biotransformation as a consequence of the nitrogen source (e.g. alachlor, metribuzin), suggesting the selective induction of certain metabolic enzymes; in other instances biotransformations were not associated with growth, suggesting secondary metabolism. An experiment was also conducted to assess the ability of *Streptomyces* (strain PS1/5) to metabolize atrazine contaminated soil. Inoculation of soil amended with 20 μg/g of atrazine and 5% chitin as carbon source resulted in ca. 78% removal of atrazine within 28 days. These data suggest that *Streptomyces* species may be potential candidates for soil inoculation to bioremediate herbicide contaminated soils.

Introduction

There is growing concern over the potential for contamination of surface and groundwater by herbicides. Although general agricultural usage probably accounts for the majority of this contamination potential, point sources (e.g. rinsate wastes from farming and ag-aviation operations) are also important, primarily because they are more likely to be in proximity to potable water supplies (wellheads). In addition, many pesticide formulating retailers may have sites sufficiently contaminated to qualify as Superfund sites (Myrick 1992). Consequently, reliable, cost effective method(s) for the remediation of herbicide contamination in soil are needed in order to minimize the potential for surface and groundwater contamination.

Biodegradation is frequently proposed as a cost effective method for the remediation of chemically contaminated sites. This approach is dependent upon either inoculation with known strains of microorganisms that are capable of degrading the contaminant molecules or the stimulation of indigenous pollutant-degrading microorganisms. Ideally, complete degradation (mineralization) of the contaminants is desirable but, at a minimum, their transformation to nontoxic products should be achieved. Relatively few soil-applied herbicides have been shown to be susceptible to mineralization by pure cultures of microorganisms. This probably is due to the fact that most herbicides contain a variety of structural groups requiring different catabolic enzyme systems which are usually not found within a single organism. In soils one likely scenario for the complete degradation of herbicides may be the initial enzymatic attack by relatively non-specific oxidases (such as the peroxidases produced

by fungi and some actinomycetes), followed by further metabolism by hydrolases and/or ring cleavage enzymes, eventually resulting in products which are mineralized via catabolic pathways. Consequently, an effective bioremediation strategy for herbicides might include the stimulation of and/or inoculation with oxidase producing microorganisms in order to accomplish the partial degradation of the parent compound to products that are degradable by other microbes.

Prior pesticide biodegradation research has demonstrated the ability of a wide variety of fungal species (e.g. *Fusarium, Aspergillus*, etc.) to nonspecifically oxidize herbicide C-C and C-Cl bonds, concomitant with growth on other carbon substrates (Kaufman & Blake 1973). More recently, the lignin-degrading white-rot fungus, *Phanerochaete chrysosporium*, has been demonstrated to fortuitously mineralize relatively persistent pesticides such as DDT and lindane (Bumpus et al. 1985), while using lignin as a growth substrate. Due to its wide diversity of oxidative enzymes, the bioremediation/bioreclamation of contaminated soils using *P. chrysosporium* has been advocated. However, the mineralization of pesticides by a single organism may be unnecessary. Previous research has shown that partial oxidation/dechlorination of recalcitrant pollutants can dramatically improve biodegradability by indigenous soil microorganisms (Hapeman-Somich 1992). Consequently, consortia of microorganisms, in which some strains are responsible for the initial oxidative attack(s) while other metabolize the oxidized products, may be just as effective in degrading/mineralizing pollutants as individual strains.

The ability of *Streptomyces* spp. to transform pesticides has not been widely investigated, despite studies by Crawford and others demonstrating the ability of *Streptomyces* to depolymerize/solubilize the lignin component of lignocellulose using oxidative enzymes (Crawford & Crawford 1980; Ramchandra et al. 1988). Bollag and co-workers have demonstrated the ability of actinomycetes and/or *Streptomyces* (strain PS1, nomenclature of Pogell; Speedie et al. 1987) to oxidize, dechlorinate and/or de-methylate metolachlor (Liu et al. 1990, 1991). Recent research by Pogell has demonstrated the ability of a derivative of PS1 (strain PS1/5) to also de-alkylate atrazine (Pogell 1992), and dechlorinate lindane (Speedie et al. 1987). In addition to their potential metabolic diversity, *Streptomyces* may be well suited for soil inoculation as a consequence of their mycelial growth habit, relatively rapid rates of growth and colonization on semi-selective substrates such as chitin (Hsu & Lockwood 1975), and their ability to be genetically manipulated (Ramchandra et al. 1987; Rowland et al. 1991; Pogell et al. 1991).

The purpose of this research was to investigate the ability of *Streptomyces* (PS1/5) to metabolize a variety of structurally different herbicides. In addition, the potential for remediation of atrazine contamined soil using PS1/5 was examined.

Materials and methods

Chemicals

The herbicides used, their concentrations and purity are shown in Table 1. The twelve herbicides represented five different classes. These were: (1) two acetanilides, alachlor (2-chloro-2′, 6′-diethyl-N-(methoxymethyl)acetanilide) and metolachlor (2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-met hylethyl) acetamide); (2) four *s*-triazines, atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), cyanazine (2-chloro-4-(1-cyano-1-methylethylamino)-6-ethylamino-s- triazine), ametryne (2-ethylamino-4-(isopropylamino)-6-(methylthio)-s-triazine), and prometryne (2,4-bis(isopropylamino)-6-methylthio-s-triazine), and the asymetric triazine, metribuzin (4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin e-5(411)-one); (3) three methyl ureas, diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea), linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea), and tebuthiuron (N-[5-(1,1-dimethylethyl)-1,3,4-thiadiazole-2-yl]-N,N′ dimethylurea); (4) one uracil, bromacil (5-bromo-3sec-butyl-6-methyluracil); and (5) one imidazole, imazapyr ((+)-2-[4,5-dihydro-4-(1-methylethyl)-5-oxo-1H-imidazole - 2-yl]-3-pyridinecarboxylic acid).

Analytical methods

Herbicides were quantified using a Waters HPLC system (Water Associates, Inc., Milford, Mass.) consisting of two 6000 pumps, a Maxima controller and data handling system, a radial compression module, a 712 WISP autosampler, and a Waters 490 programmable multi-wavelength detector. Separation was achieved by using a radially compressed cartridge (C-18 Novapack 4 μm, Waters). The mobile phase for the various pesticides and the wavelength monitored for detection are listed in Table 1. The flow rate was 2.0 ml/min. Recoveries are expressed as the percentage remaining,

Table 1. Concentrations, analytical conditions, and purity of the twelve herbicides used in this study.

Pesticide	Initial concentration (μg/ml)	Detection Wavelength (nm)	Mobile phase (%) ACN	(%) water	Purity
Alachlor	50	210	50	50	99
Ametryne	47	230	50	50	98
Atrazine	27	225	30	70	96
Bromacil	50	275	25	75	99
Cyanazine	105	230	25	75	T.G.
Diuron	50	260	30	70	T.G.
Imazapyr	39	235	10	90	98
Linuron	49	260	40	60	T.G.
Metolachlor	35	210	50	50	95
Metribuzin	58	230	20	80	T.G.
Prometryne	32	230	50	50	T.G.
Tebuthiuron	33	255	25	75	99

T.G. = Technical Grade (> 95%); precise purity unknown.

using the initial concentration (day 0) as a baseline (100%).

Aqueous incubations

The growth media consisted of the herbicide dissolved in deionized water (initial concentrations are listed in Table 1), 1% dextrin as the carbon source, 20 mM phosphate buffer, trace metals solution (Shelton & Somich 1988), and either 0.5% vitamin-free Casamino-acids (CAS) or 40 mM NH_4^+ (ammonium sulfate) as the N-source. The pH was adjusted to ca. 7.0 and the solution filter sterilized using 0.2 micron filters. The sterilized solution (100 ml) was placed in sterile 250 ml baffled flasks, inoculated with *Streptomyces* (strain PS1/5; Speedie et al. 1987), and shaken on a rotary shaker at 30° C. Incubations were conducted in duplicate; an uninoculated control was included for each herbicide to assess abiotic losses.

Herbicide concentrations were routinely monitored by combining 0.75 ml of culture with 0.75 ml of acetonitrile, shaking vigorously, and storing in glass vials at - 4° C until analysis. After thawing, samples were shaken vigorously, centrifuged at high speed in a microcentrifuge, and the supernatant analyzed by HPLC.

In order to determine whether binding of herbicides to microbial biomass was rendering them non-extractable, extraction efficiencies were determined independently by comparing recoveries in the absence or presence of biomass. One ml of *Streptomyces* PS1/5 culture in late log phase was centrifuged and the supernatant decanted, then 0.75 ml of herbicide solution was added and the mixture incubated for 30 min. The culture was then extracted as previously described.

Soil incubations

Soil was obtained from field plots located at the Beltsville Agricultural Research Center, South Farm (Beltsville, MD). The soil was a Hatboro silt loam (fine-loamy, mixed, nonacid, mesic Typic Fluvaquents) with 1.2% organic matter and pH of 6.05. Soils were air-dried, crushed, and sieved prior to use.

Soil (450 g) mixed with chitin (50 g) (practical grade from crab shells; Sigma, St. Louis, MO) was combined with an atrazine solution (11 mg of Aatrex dispersible granules in 150 ml water) using a slurry technique (Shelton & Parkin, 1989) to give a final gravimetric water content of 30% (ca. - 0.3 bar) and 20 μg atrazine g^{-1} dry weight soil. Soils were inoculated with ca. 10 g (wet weight) of fresh, pelleted *Streptomyces* PS1/5 (unwashed) cultured in dextrin-tryptone medium. Duplicate soil flasks were incubated at 25° C with a constant air flow (ca. 30 ml min^{-1}) to dissipate excess ammonia. Periodically, 20 g of soil/chitin from the flasks was combined with 50 ml of acetonitrile, shaken for 1 hr on a wrist action shaker, and

the supernatant analyzed by HPLC. A sterile control flask, containing 0.4 ml of a 20% azide solution, was included to assess abiotic atrazine losses. Recoveries are expressed as the percentage remaining using the initial concentration (day 0) as a baseline (100%).

Results and discussion

There was no evidence for abiotic losses of herbicides in aqueous culture; concentrations in uninoculated controls were constant, or increased slightly due to water evaporation (data not shown). Extraction efficiencies from biomas (30 min incubation) varied from 95 to 100%, indicating that losses of herbicides were not due to adsorption to mycelial biomass.

Microscopic examination of cultures during the course of the incubation indicated a rapid rate of growth with maximum biomass on day 3–5, followed by equally rapid rates of cell lysis. Rates and extent of biomass production were comparable for CAS and NH_4^+ (data not shown).

Streptomyces (PS1/5) transformed to varying degrees all twelve herbicides tested, representing five classes of herbicides, and possessing a wide range of functional groups. Both acetanilides (alachlor and metolachlor) were metabolized by strain PS1/5 (Fig. 1). Rates of biotransformation of acetanilides were more rapid with CAS than with NH_4^+ as the N-source; this effect was most pronounced with alachlor. Percent biotransformation after 7 days was 95% (CAS) vs. 59% (NH_4^+) for alachlor and 96% (CAS) vs. 80% (NH_4^+) for metolachlor. Our results with metolachlor are consistent with those of Liu et al (1991) who observed rapid transformation of metolachlor with strain PS1. Liu identified seven products resulting from the dechlorination of metolachlor. In the present study, it is unclear whether only dechlorination occurred or whether hydroxylation and/or demethylation also occurred. Recent work by Pogell (1992) has established the ability of this strain to de-alkylate, presumably via an oxidative mechanism. In addition, Krause et al. (1985) identified eight metabolites from metolachlor, including various combinations of aralkyl hydroxylated and N-alkyl demethylated products, produced by an unidentified soil actinomycete. Rates for alachlor were similar to metolachlor; presumably biotransformations were catalyzed by the same enzyme(s).

All four *s*-triazines were metabolized by strain PS1/5 (Fig. 2). Ametryne and prometryne were rapidly transformed; percent biotransformation after 7 days was 97% for ametryne and 100% for prometryne, regardless of the N-source. Atrazine and cyanazine were also transformed although at slower rates; percent biotransformation after 7 days was 59% (CAS) vs. 83% (NH_4^+) for atrazine and 47% (CAS) vs. 32% (NH_4^+) for cyanazine. The affinity of PS1/5 for cyanazine appeared to be significantly lower, although the mass of cyanazine degraded (30–45 μg/ml) was actually greater than that of atrazine. Pogell (1992) identified three metabolites produced from atrazine by PS1/5: in order of prevalence, de-isopropylatrazine (CEAT), de-ethylatrazine (CIAT), and de-alkylatrazine (CAAT). Peaks corresponding to the retention times for these products were observed in HPLC chromatograms but were not quantified (data not shown).

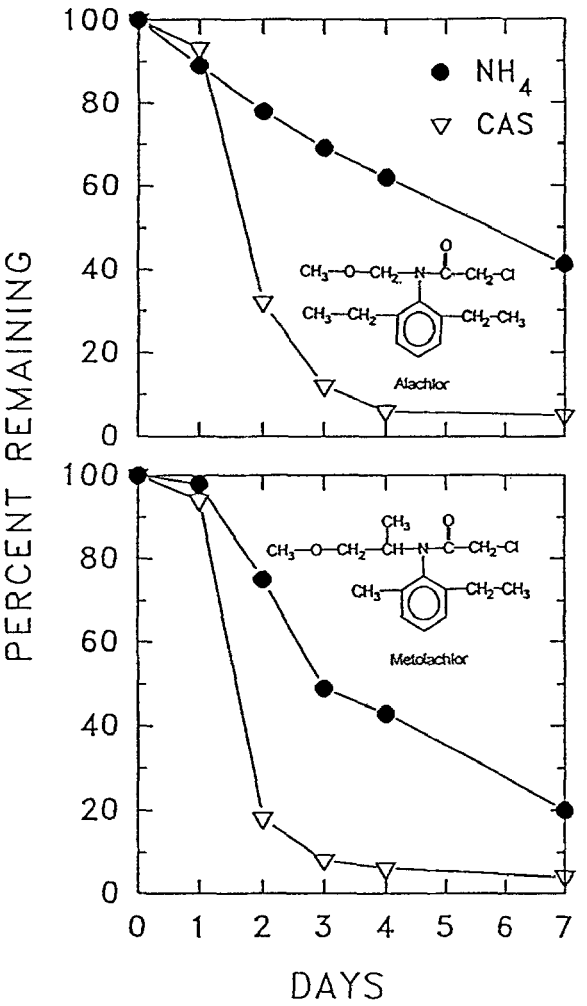

Fig. 1. Metabolism of the acetanilide herbicides alachlor and metolachlor by *Streptomyces* (strain PS1/5) as a function of nitrogen source (casamino acids vs. ammonium).

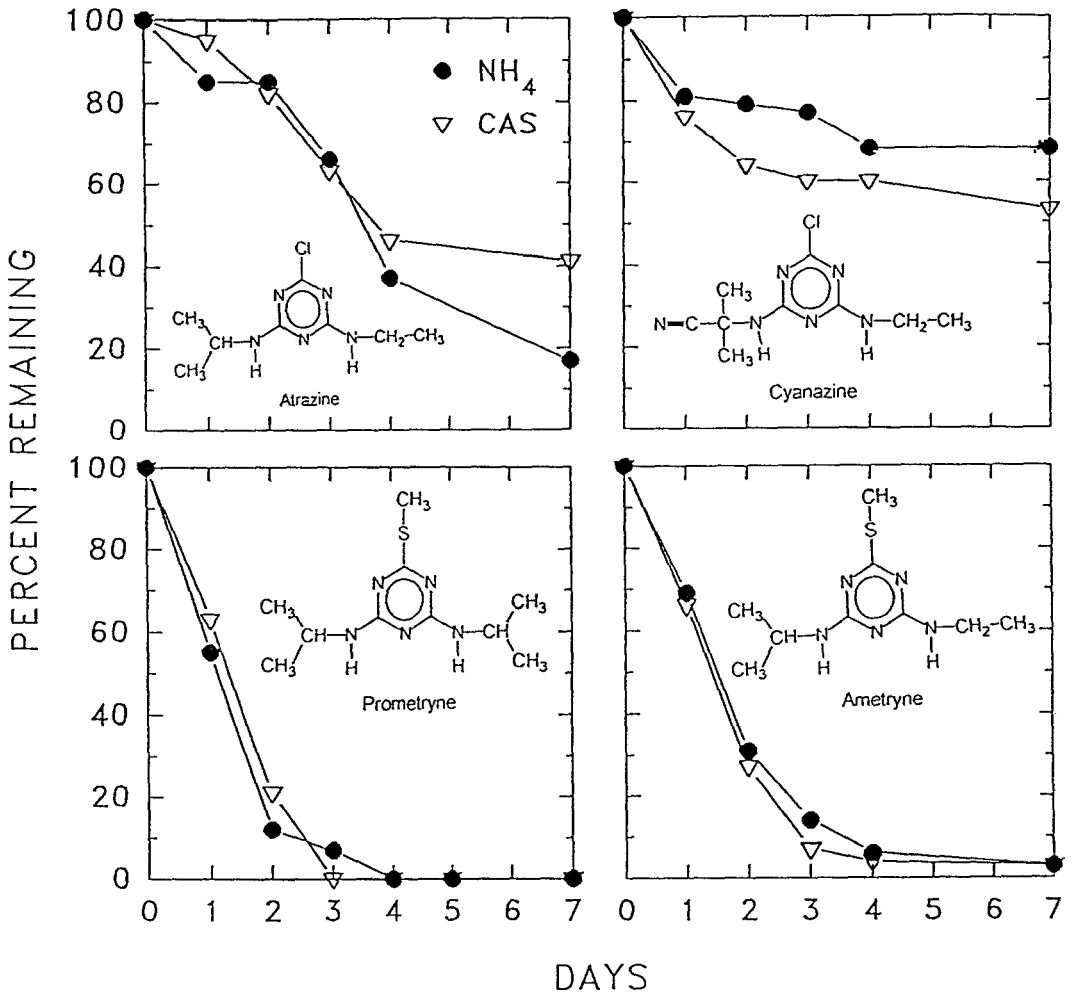

Fig. 2. Metabolism of the *s*-triazine herbicides atrazine, cyanazine, ametryne, and prometryn by *Streptomyces* (strain PS1/5) as a function of nitrogen source (casamino acids vs. ammonium).

Two of the three methyl ureas were rapidly metabolized by strain PS1/5 (Fig. 3). Results for linuron and tebuthiuron were comparable; rates of transformation were relatively rapid regardless of N-source. Percent biotransformation after 7 days was 80% (CAS) vs. 94% (NH_4^+) for linuron and 67% (CAS) vs. 85% (NH_4^+) for tebuthiuron. By comparison, diuron was poorly metabolized; rates of biotransformation (with NH_4^+ as the N-source) was greatest during the time when cells were lysing. Percent biotransformation after 7 days was 19% (CAS) vs. 55% (NH_4^+).

Imazapyr (imidazole) and metribuzin (asymetric triazine) were poorly metabolized (Fig. 4). There was no evidence for transformation of either compound with CAS as the N-source; percent biotransformation after 7 days with NH_4^+ as N-source was 48% for imazapyr and 40% for metribuzin. Bromacil (uracil) was rapidly transformed, regardless of the N-source; the lag in transformation was due to a low initial inoculum (Fig. 4). Percent biotransformation after 7 days was 92% (CAS) vs. 77% (NH_4^+).

Although these kinetic data are strictly qualitative, the general patterns of metabolism clearly suggest different activities and/or affinities. Rates and extent of triazine biotransformations were dramatically stimulated when the Cl substituent was replaced with a thiomethyl moiety, indicating a greatly enhanced affinity for these compounds, and/or metabolism of the thiomethyl moiety. Rates and extent of alachlor and metolachlor biotransformation were enhanced with CAS as the N-source, suggesting the expression of separate, or different, enzymes in the presence of an adequate exogenous source of amino acids. This was not observed with other herbicides, indicating either that alachlor or

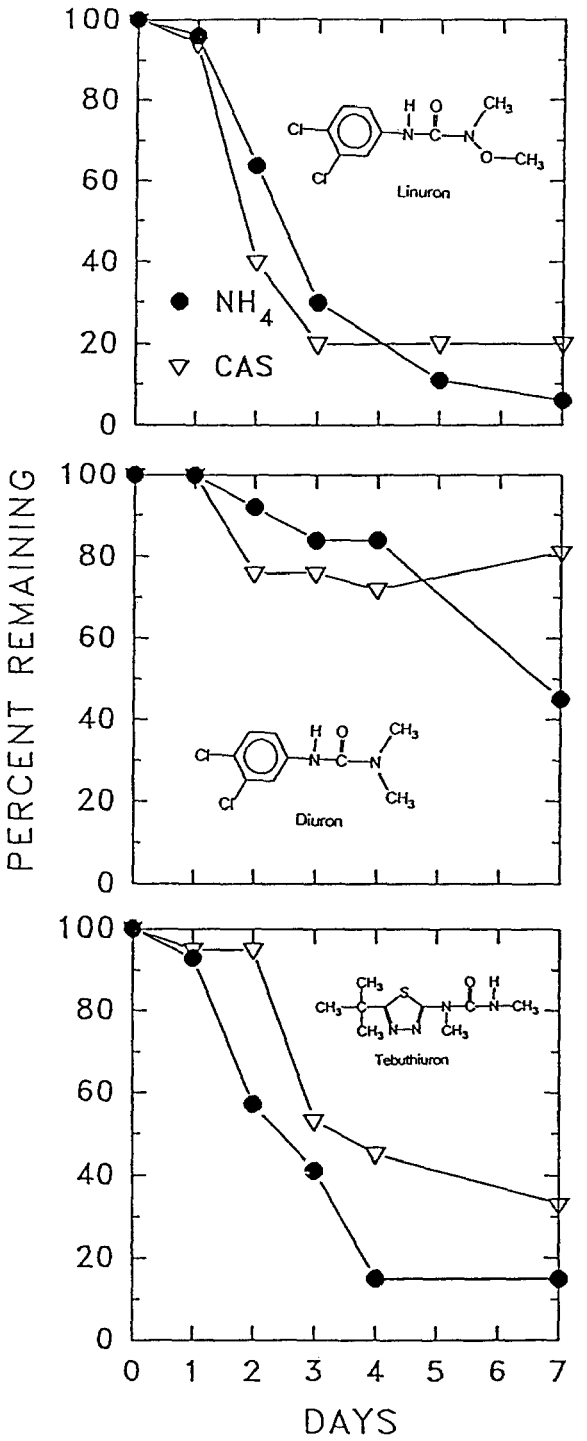

Fig. 3. Metabolism of the urea herbicides linuron, diuron, and tebuthiuron by *Streptomyces* (strain PS1/5) as a function of nitrogen source (casamino acids vs. ammonium).

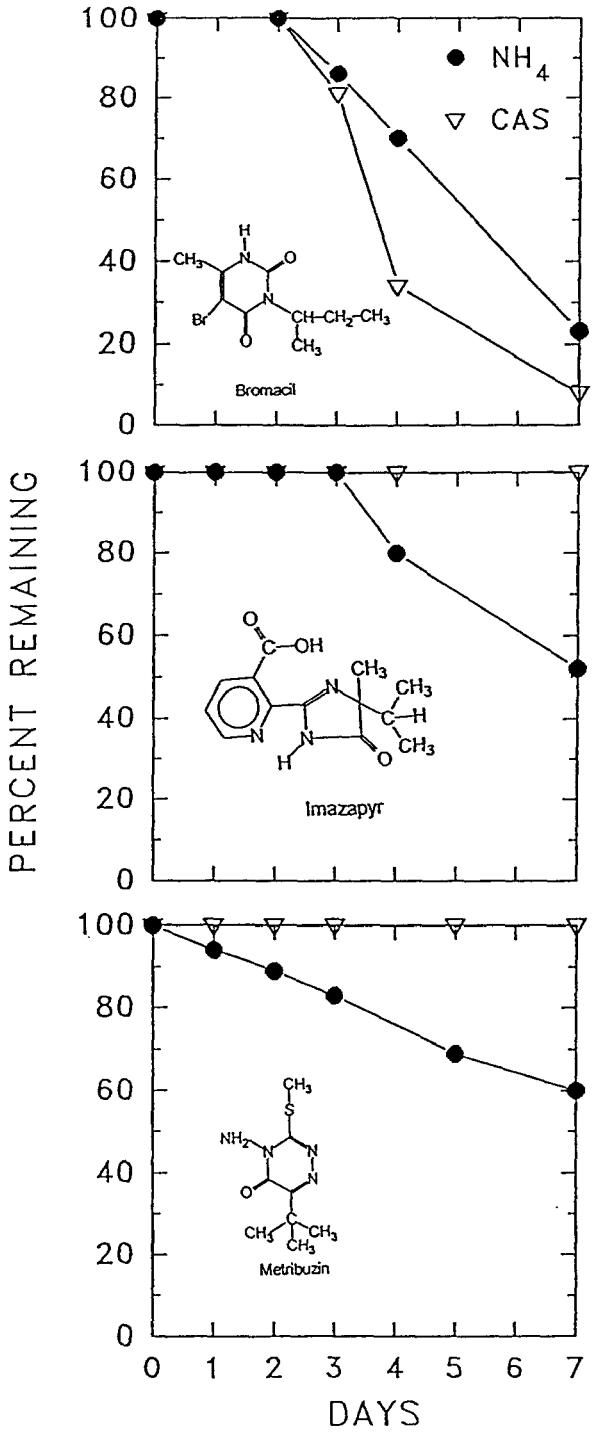

Fig. 4. Metabolism of the asymmetric herbicide metribuzin, imidazole herbicide imazapyr, and uracil herbicide bromacil by *Streptomyces* (strain PS1/5) as a function of nitrogen source (casamino acids vs. ammonium).

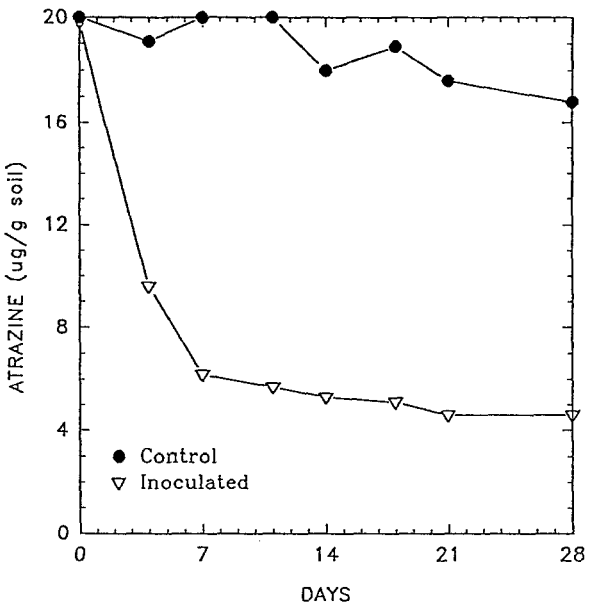

Fig. 5. Metabolism of atrazine in soil (20 μg/g) amended with 5% chitin (by weight); initial soil moisture was ca. - 0.3 bar.

metolachlor were specific inducers of the biochemical system that affected them, or that certain transformations were unique to acetanilides. By comparison, rates and extent of metribuzin and imazapyr biotransformations appeared to be inhibited by the presence of amino acids. For several herbicides, rates of biotransformation with NH_4^+ as N-source appeared to be linear or to increase with cell lysis (e.g. alachlor, diuron, metribuzin, imazapyr). This pattern is inconsistent with growth kinetics, and suggests that enzymes associated with secondary metabolism may have been involved.

Polar products were frequently observed in HPLC chromatograms concomitant with the disappearance of parent compounds; these products were not identified. This is consistent with previous research which indicates that *Streptomyces* strains are primarily responsible for the initial transformation, as opposed to mineralization, of organic pollutants.

Inoculation of soil (plus 10% chitin) with *Streptomyces* (strain PS1/5) resulted in extensive transformation of atrazine; approximately 78% of atrazine was biotransformed after 28 days (Fig. 5). Atrazine concentrations in the sterile control were stable throughout most of the incubation, indicating losses of atrazine due to volatilization were insignificant. Although no attempt was made to quantify chitin metabolism, chitin flakes appeared to 'liquify' over the course of the incubation, indicating extensive metabolism. In addition, microscopic examination of chitin flakes indicated rapid colonization. The final atrazine concentration in soil (ca. 4.5 μg/g) was comparable with the final atrazine concentration in aqueous culture (ca. 4.8 μg/ml), suggesting that this may represent the lower limit of atrazine metabolism by strain PS1/5. Although a higher affinity for atrazine would be preferable, these data suggest that *Streptomyces* strains may be viable candidates for soil inoculation.

Conclusions

Since most pesticide mixing sites are contaminated with a wide variety of herbicides, any potential inoculant used in bioremediation/bioreclamation must be able to degrade a variety of structurally diverse pesticides. In addition, the ability to survive and proliferate in contaminated soils is mandatory. In conjunction with previous studies, these data suggest that *Streptomyces* may have sufficient metabolic diversity to be useful as a soil inoculant. In addition, the soil inoculation data suggest that bioremediation may be feasible using semi-selective substrate/carriers, such as chitin. The ability of many *Streptomyces* strains to be genetically engineered is another attractive feature. Further research is necessary, however, in order to elucidate metabolic pathways, induction/expression phenomena, and for strain improvement.

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