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ORIGINAL PAPER

Edward Topp

A comparison of three atrazine-degrading bacteria for soil bioremediation

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Abstract The ability of three atrazine-degrading bacteria, *Pseudomonas* sp. strain ADP, a *Pseudaminobacter* sp., and a *Nocardioides* sp., to degrade and mineralize this herbicide in a loam soil was evaluated in laboratory microcosms. These bacteria all hydrolytically dechlorinate atrazine, and degrade atrazine in pure culture with comparable specific activities. The *Pseudaminobacter* and *Nocardioides* can utilize atrazine as sole carbon and nitrogen source, whereas the *Pseudomonas* can utilize the compound only as a nitrogen source. The *Pseudomonas* and *Pseudaminobacter* mineralize the compound; the end product of atrazine metabolism by the *Nocardioides* is N-ethylammelide. At inoculum densities of 10^5 cells/g soil, only the *Pseudaminobacter* and *Nocardioides* accelerated atrazine dissipation. The *Pseudaminobacter* mineralized atrazine rapidly and without a lag, whereas atrazine was mineralized in the *Nocardioides*-inoculated soil but only after a lag of several days. The *Pseudaminobacter* remained viable longer than did the *Pseudomonas* in soil. PCR analysis of recovered bacteria indicated that the genes *atz*A (atrazine chlorohydrolase) and *atz*B (hydroxyatrazine ethylaminohydrolase) were less stable in the *Pseudaminobacter* than the *Pseudomonas*. In summary, this study has revealed important differences in the ability of atrazine-hydrolyzing bacteria to degrade this compound in soil, and suggests that the ability to utilize atrazine as a carbon source is important to establish "enhanced degradation" by ecologically meaningful inoculum densities.

Keywords Atrazine · Bioremediation · Pesticide degradation · Biodegradation

E. Topp (✉)
Agriculture and Agri-Food Canada,
Southern Crop Protection and Food Research Center,
1391 Sandford St., London, ON, N5V 4T3, Canada
e-mail: toppe@em.agr.ca
Tel.: +1-519-4571470, Fax: +1-519-4573997

Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) has been on the market for about 50 years, and is used extensively in many parts of the world to control a variety of weeds, primarily in the production of maize (Tomlin 1994). This herbicide is a suspected endocrine-disrupting chemical, frequently detected in drainage water in cultivated areas, and is relatively stable in groundwater (Agertved et al. 1992; Moore and Waring 1998). Historically, atrazine persistence in soils was characterized by a moderate half-life (DT_{50}) of about 35–50 days with very little mineralization of the *s*-triazine ring. Recently, however, rapid atrazine mineralization has been detected in North American and European agricultural soils that have come in contact with the herbicide in normal farming practice (Pussemier et al. 1997; Houot et al. 2000). The ready isolation of atrazine-degrading bacteria from these soils attests to the biological basis for this rapid degradation (Mandelbaum et al. 1995; Topp et al.2000a, b) These bacteria commonly initiate atrazine degradation by hydrolytic dechlorination (Fig. 1). The genes encoding an atrazine chlorohydrolase (*atzA*) and two amidohydrolytic reactions (*atzB* and *atzC*), which together convert atrazine to the ring-cleavage substrate cyanuric acid, have been cloned from the *Pseudomonas* sp. strain ADP (de Souza et al. 1995; Boundy-Mills et al. 1997; Sadowsky et al. 1998). Cyanuric acid is converted by another set of amidohydrolase enzymes to biuret and urea, which are then mineralized (Cook et al. 1985). The genes encoding these enzymes are widespread, highly conserved, plasmid-borne, and in some cases associated with catabolic transposons (Karns and Eaton 1997; de Souza et al. 1998a, b; Topp et al. 2000a).

A number of investigators have explored the utility of pure or mixed cultures of atrazine-degrading bacteria for bioremediating soils, groundwater or waste streams contaminated with atrazine (Alvey and Crowley 1996; Assaf and Turco 1994; Grigg et al.1997; Shapir and Mandelbaum 1997; Crawford et al. 1998; Struthers et al. 1998; Newcombe and Crowley 1999; Strong et al. 2000). Both

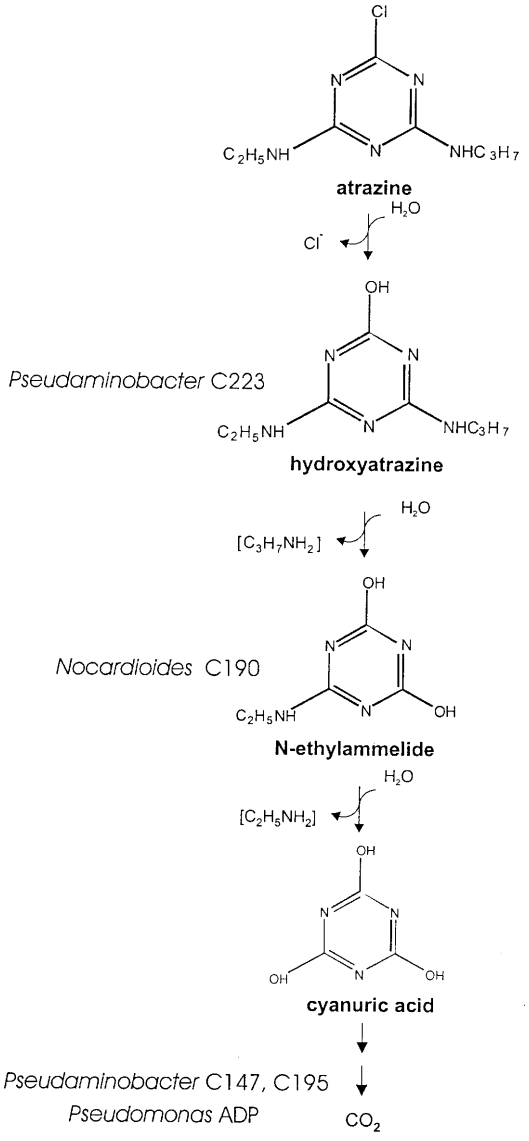

Fig. 1 Pathways of atrazine metabolism by the bacteria used in this study. The molecule adjacent to the strain designation is the end product of atrazine metabolism for that strain

field and laboratory trials with a range of matrices and atrazine concentrations have generally been successful, although high inoculum densities and frequent re-inoculation may be required, which is a significant economic and practical consideration. Traits which enhance the activity of bioremediation agents in soils are therefore desirable.

The work described here compared three atrazine-degrading bacteria, *Pseudomonas* sp. strain ADP, a *Pseudaminobacter* sp., and a *Nocardioides* sp. for the ability to degrade and mineralize this herbicide in soil.

Materials and methods

Bacteria

The bacteria used in this study were *Pseudomonas* sp. strain ADP, a *Pseudaminobacter* sp. (wild type strains C147 or C195; and strain C223, a spontaneous *atzB*$^-$ mutant of strain C195) and a *Nocardioides* sp. strain C190. Some characteristics of these bacteria are presented in Table 1, and Fig. 1 illustrates the pathway of atrazine metabolism by each isolate. Further detailed descriptions of these organisms can be found in Mandelbaum et al. (1995), and Topp et al. (2000a, b). The ability to denitrify was established by testing for growth and gas production at the bottom of stabs prepared with nutrient broth yeast extract agar (NBYEA) supplemented with 1 g/l sodium nitrate and 1 g/l glucose and incubated at 30°C. Bacteria were stored frozen in 15% glycerol at –70°C. Cells for soil inoculation experiments were routinely grown at 30°C on solidified atrazine mineral salts (AMS) medium containing atrazine as the sole nitrogen source and citrate and methanol as additional carbon sources (Topp et al. 2000a). None of the bacterial strains originate from the soil used in this study, and therefore none had an inherent advantage in this respect.

Soil microcosm incubations

A loam soil (pH 7.30; 3.5% organic matter; 48% sand, 43% silt and 9% clay) from the Southern Crop Protection and Food Research Center farm near London, Ontario, Canada was used in all experiments. The soil was under sod, and had no atrazine treatment history. Soils were sieved to a 2-mm maximum particle size and stored at 4°C until used. In some cases 1-kg portions of soil were sterilized by autoclaving for 1 h, incubating at room temperature for 24 h, and autoclaving for an additional hour.

Fifty or 100-g (moist weight, adjusted to a moisture of 15% unless otherwise indicated) portions of soil were added to 150-ml babyfood jars. Herbicide was added to the soil without solvent carryover by adding methanol stock solutions of unradiolabelled atrazine and [*ring*-U-^{14}C]atrazine to 2-g portions of ground air-dried soil, allowing the methanol to evaporate, and thoroughly incorporating this material into the moist soil. Unless otherwise indicated, the initial atrazine concentration was 1 mg/kg dry soil, and the initial radioactive concentration was 5,000 dpm/g. The jars were placed in sealable 1-l mason jars containing two scintillation vials, one containing water to maintain moisture in the jar headspace, and the other containing 7 ml 1 N NaOH as a CO_2 trap. Jars were incubated at 30°C for 24 h prior to inoculation with atrazine-degrading bacteria. Bacteria recovered from 2-week-old AMS plates were resuspended in sterile water to a density of about 1×10^8 colony forming units (CFU)/ml (adjusted using a standard curve relating absorbance at 600 nm with plate count on Tryptic Soy Agar or NBYEA). The cell density was adjusted so that thorough incorporation of 2.5 ml cell suspension would inoculate the soil with 1×10^5 or 1×10^7 atrazine-degrading bacteria/g dry soil as required. Uninoculated controls received 2.5 ml sterile water. The CO_2 traps were replaced periodically, and the recovered radioactivity measured by liquid scintillation counter (LSC). Five gram portions of soil were removed periodically for chemical or microbiological analysis. Samples for microbiological analysis were processed immediately, and samples for chemical analysis were stored at –20°C until analyzed. All treatments were in triplicate. All data are reported as means ± standard deviation.

Chemicals and analytical methods

Analytical grade triazine herbicides and metabolites were gifts from Novartis Crop Protection Canada (Guelph, ON, Canada) or were purchased from Chem Service Inc (West Chester, Pa.; see Fig. 1 for structures). [*ring*-U-^{14}C]Atrazine (specific activity 4.5 mCi/mmol, radioactive purity 95%) was purchased from Sigma (St. Louis, Mo.). Atrazine and transformation products were ana-

Table 1 Characteristics of the bacteria used in this study[a]

Parameter	*Pseudaminobacter* sp. strains C147 or strain C195	*Pseudaminobacter* sp. strain C223	*Pseudomonas* sp. strain ADP	*Nocardioides* sp. strain C190
Mechanism of atrazine transformation	Hydrolytic dechlorination	Hydrolytic dechlorination	Hydrolytic dechlorination	Hydrolytic dechlorination
Regulation of atrazine transformation	None known	None known	Repressed by high inorganic nitrogen concentrations	None known
Genes encoding 'upper pathway'	*atzABC*	*atzAC*, *atzB* lost spontaneously	*atzABC*	Not *atzABC*
End product of triazine ring-carbon	CO_2	Hydroxyatrazine	CO_2	N-ethylammelide
Use of atrazine as N source	Yes	No	Yes	Yes
Use of alkylamine side chains as C source	Yes; both ethyl- and isopropylamine	No	No	Yes; isopropylamine only
K_m, V_{max} of atrazine chlorohydrolase	Not done	Not done	149 µM, 2.6 µmol/min mg protein	25 µM, 31 µmol/min mg protein
Denitrification	Yes	Yes	No	No

[a]Information for this table was drawn from this study, Mandelbaum et al. 1995; Topp et al. 2000a; b

lyzed by reverse phase HPLC on a C-18 column using an instrument equipped with a UV detector (set at 220 nm) coupled in series with a radioactivity detector (Topp et al. 2000a). The solvent consisted of 70% methanol:30% 5 mM Na_2HPO_4 (pH 9.0; solvent system 1), or 50% methanol:50% 10 mM ammonium acetate (solvent system 2). The radioactivity of the samples was measured in Universol scintillation cocktail (ICN, Cosa Mesa, Calif.) with a Beckman model LS5801 (Irvine, Calif.) LSC using an external standard for quench correction. Soil extracts were prepared for analysis by shaking 5-g soil samples for 1 h with two 5-ml portions of methanol, pooling the extracts, taking to dryness under a stream of nitrogen, resuspending in 500 l methanol, centrifuging the extract (3 min, 14,000*g*), and recovering the supernatant for analysis.

Enumeration of inoculated bacteria, and assessment of the stability of the atrazine degrading phenotype in soil

Viable soil populations of *Pseudomonas* strain ADP and of *Pseudaminobacter* strain C147 were determined by plating serial soil dilutions (prepared in 2 $g \cdot l^{-1}$ sodium metaphosphate) on semi-selective media consisting of NBYEA supplemented with 50 mg/l cycloheximide, and either 20 mg/l ampicillin (for *Pseudomonas* ADP) or 50 mg/l kanamycin (for *Pseudaminobacter* C147). In preliminary experiments the addition of the antibiotics to NBYEA did not reduce the plating efficiency of pure cultures (data not shown). The specificity of the medium was validated in preliminary experiments by subjecting 36 colonies of presumptive (on the basis of colony morphology) *Pseudomonas* ADP and of *Pseudaminobacter* C147 recovered from soil plate counts to rep-PCR with the BOXA1R and ERIC primers as described in Topp et al. (2000a) The rep-PCR method has a high level of phylogenetic precision, and bacteria which have identical fingerprinting patterns can reasonably be expected to be siblings. All the isolates had rep-PCR fingerprints corresponding to the inocula, indicating that the inoculant strains could be identified on the basis of colony morphology with a high degree of confidence. The detection limit was approximately 10^4 inoculated bacteria/g soil.

The stability of genes encoding atrazine chlorohydrolase (*atz*A) and hydroxyatrazine ethylaminohydrolase (*atz*B) in the inoculated populations was determined using a PCR method. At each sampling time 36 colonies recovered from soil on the semi-selective media were subjected to rep-PCR using the BOXA1R and ERIC primers to confirm identity. The presence of *atz*A in these recovered bacteria was determined by PCR using primers specific for this gene (de Souza et al. 1998b) which yielded a product of about 500 bp, and by showing clearing on atrazine-containing plates (Mandelbaum et al. 1995). Preliminary experiments showed complete concordance between the PCR method and the plate clearing assay. The presence of *atz*B was established by PCR with the primers 5′-TCACCGGGGATGTCGCGGGC-3′ and 5′-AGACTCGACGAAGGTT-3′, which yielded the expected product of about 650 bp from all strains carrying *atz*B. The specificity of the primers was shown through the absence of a product from *Pseudaminobacter* sp. strain C223 which has spontaneously lost *atz*B (Topp et al. 2000a). The PCR reaction mixture composition and thermocycler program were as previously described (Topp et al. 2000a) except that the template consisted of whole cells resuspended in distilled water to an A_{600} of 1.

Results

Cells of *Pseudomonas* strain ADP, *Pseudaminobacter* strain C147 and *Nocardioides* strain C190 degraded atrazine at comparable rates when resuspended to the same cell density in AMS medium containing 10 mg atrazine/l as the sole source of carbon and nitrogen (Fig. 2). The *atzB*– mutant *Pseudaminobacter* strain C223 degrades atrazine at rates comparable to the wild-type strains *Pseudaminobacter* C147 and C195, and accumulates stoichiometric quantities of hydroxyatrazine in AMS medium (Topp et al. 2000a). The bacteria did vary with respect to the accumulation of hydroxyatrazine during the incubation. *Pseudomonas* ADP did not produce any detectable hydroxyatrazine, *Pseudaminobacter* C147 converted a fraction of the atrazine to this product, and *Nocardioides* C190 accumulated stoichiometric amounts. In longer incubations or at higher cell density, the *Pseudaminobacter* fully mineralized the s-triazine ring, whereas the *Nocardioides* accumulated stoichiometric amounts of the end product N-ethylammelide (Topp et al. 2000a, b).

Atrazine dissipation by the two atrazine-mineralizing Gram-negative bacteria, and the *Nocardioides* sp. was

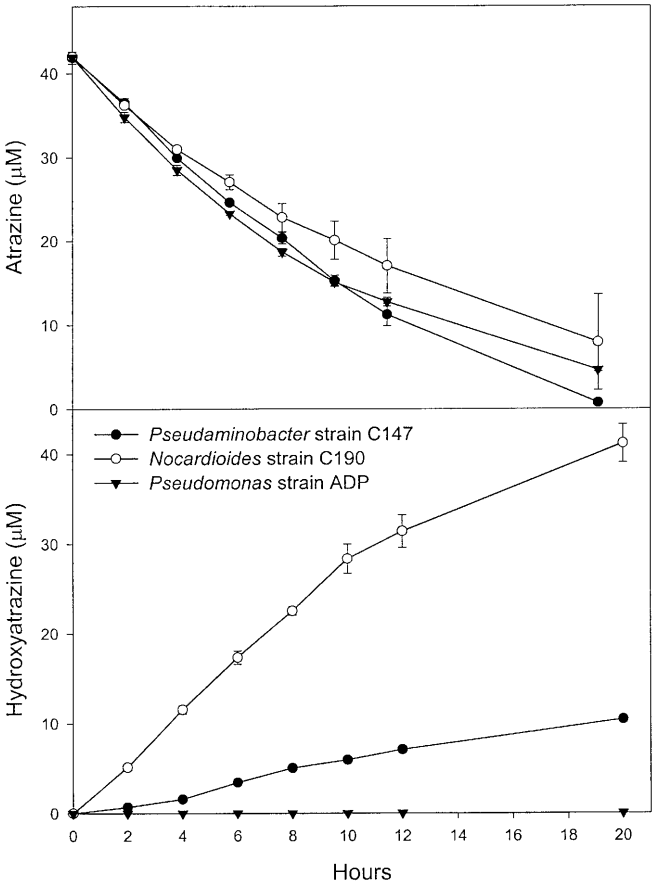

Fig. 2 Atrazine degradation by *Pseudaminobacter* C147, *Pseudomonas* ADP, and *Nocardioides* C190 in a mineral salts medium. Cells were resuspended to an initial optical density (600 nm) of 0.1 in mineral salts medium containing 10 mg atrazine/l as the sole source of carbon and nitrogen

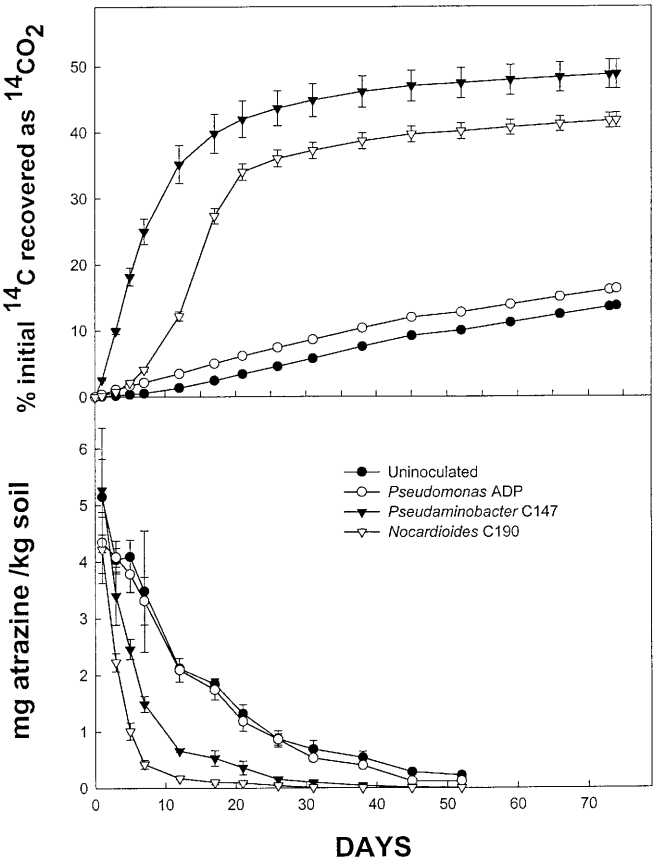

Fig. 3 Atrazine dissipation (*lower panel*) and mineralization (*upper panel*) in soil. Bacteria were inoculated at an initial density of 10^5 cells/g soil

investigated in soil inoculated with 10^5 cells/g (Fig. 3). The *Pseudaminobacter* and *Nocardioides*, but not the *Pseudomonas* accelerated atrazine removal compared to the uninoculated control. The half life of atrazine was about 10 days in the uninoculated and *Pseudomonas*-inoculated soil, about 5 days in the *Pseudaminobacter*-inoculated soil, and about 3 days in the *Nocardioides*-inoculated soil. [*Ring*-U-^{14}C]Atrazine was rapidly mineralized without a lag in soil inoculated with the *Pseudaminobacter*. In uninoculated soil, atrazine was slowly and linearly mineralized with final $^{14}CO_2$ accumulated representing only 5% of the initial radioactivity added. There was no significant enhancement of atrazine mineralization rates in the *Pseudomonas*-inoculated soil. *Pseudaminobacter* strain C223, a mutant which has spontaneously lost *atzB* and converts atrazine stoichiometrically to hydroxyatrazine, did not stimulate atrazine degradation or mineralization when inoculated into the loam soil (data not shown).

The *Nocardioides*, itself unable to mineralize atrazine, promoted atrazine mineralization to rates and yields comparable to those in the *Pseudaminobacter*-inoculated loam soil, but only after a lag of approximately 7 days. The *Nocardioides* did not stimulate atrazine mineralization when inoculated into sterile soil (data not shown).

The survival of viable cells was determined by plating inoculated soil samples on a semi-selective medium (data not shown). It was necessary to inoculate the soils heavily in these experiments, 10^7 cells/g, because of the enumeration method's relatively high detection limit. The decline in viable populations of both organisms was generally close to first order (the r^2 of linear regression of log-transformed data was 0.9448 for the *Pseudomonas*, 0.9829 for the *Pseudaminobacter*). Loss of viable populations was more pronounced for the *Pseudomonas* (half life of 3.0 days) than the *Pseudaminobacter* (half life of 5.5 days).

The stability of the atrazine-degradation phenotype in bacteria inoculated into soil was assessed by testing isolates recovered from soil for the presence or absence of *atz*A and *atz*B (data not shown). At the start of the experiment, 92% of the *Pseudomonas* sp. inoculum possessed *atz*A, and 97% possessed *atz*B. In contrast, 81% of the *Pseudaminobacter* sp. inoculum possessed *atzA* whereas only 66% possessed *atz*B. In populations recovered from the soil after 2 weeks of incubation, about

80% of the *Pseudomonas* sp. isolates possessed *atz*A and *atz*B, whereas 46% of the *Pseudaminobacter* sp. isolates possessed *atz*A, and only 29% *atz*B.

Discussion

This study revealed important differences in the efficacy of atrazine-degrading bacteria for soil bioremediation. Perhaps the most noteworthy result was the difference between *Pseudomonas* ADP and the *Pseudaminobacter* sp. (Fig. 3). Both these organisms degrade atrazine at comparable rates in pure culture, convert atrazine to the ring cleavage substrate cyanuric acid by means of *atz*ABC, and mineralize ring-labelled atrazine (Table 1). Both organisms can utilize atrazine as a nitrogen source. However, a key difference between these organisms is that only the *Pseudaminobacter* sp. can use atrazine as a carbon source. The *Pseudaminobacter* sp. can utilize both ethylamine and isopropylamine, produced during the conversion of hydroxyatrazine to cyanuric acid, as carbon source (Topp et al. 2000a). It is reasonable to hypothesize that the more rapid degradation and mineralization of atrazine, and robust viability of the *Pseudaminobacter* in comparison with the *Pseudomonas* in soil is due to the ability of the latter to utilize atrazine as a carbon source. This hypothesis is substantiated by the observation that the *atzB*$^-$ mutant *Pseudaminobacter* C223 does not enhance atrazine degradation when added to soil. Furthermore, the *Nocardioides*, which can likewise utilize atrazine as a carbon source, accelerated atrazine degradation. Population dynamics were not followed in these experiments. However, the immediate atrazine dissipation and good fit to first-order kinetics (for example the log transform of atrazine dissipation in *Pseudaminobacter* C147-inoculated soil had an r^2 of 0.9879, Fig. 3) suggest that the dissipation kinetics were not influenced by growth. More direct evidence for the impact of atrazine-carbon utilization on its removal would come from a comparison of the efficacy of mutants of the *Pseudaminobacter* sp. compromised in the ability to utilize ethylamine and isopropylamine, with their isogenic parent. However, this bacterium has proved recalcitrant to transposon mutagenesis by conjugative mating or electroporation.

The *Pseudomonas* strain ADP was ineffective at an initial inoculum density of 10^5 cells/g soil, but all three wild-type bacteria degraded atrazine rapidly at comparable rates when inoculated into the loam at a density of 10^7 cells/g (data not shown). A number of studies have shown that atrazine-degrading bacteria which are unable to use the compound as a carbon source can enhance atrazine bioremediation when added at relatively high inoculum levels, or when readily metabolizable carbon sources are provided to support growth. (Mandelbaum et al. 1995; Shapir and Mandelbaum 1997; Newcombe and Crowley 1999). On the other hand, *Agrobacterium radiobacter* J14a, which can utilize the ethylamine sidechain carbon atoms, enhanced atrazine degradation when added to soil at 10^5 cells/g (Struthers et al. 1998). Taken together, these results suggest that the ability to utilize the target pollutant as a carbon source can enhance the efficacy of a bioremediation agent.

The mineralization of [*ring*-U-^{14}C]atrazine in non-sterile but not in sterile soil inoculated with *Nocardioides* C190 indicated that other soil microorganisms are able to metabolize transformation products containing the *s*-triazine ring. Both [*ring*-U-^{14}C]hydroxyatrazine (recovered from cultures of *Pseudaminobacter* C223 incubated with [*ring*-U-^{14}C]atrazine) and [*ring*-U-^{14}C]N-ethylammelide (recovered from cultures of *Nocardioides* C190 incubated with [*ring*-U-^{14}C]atrazine) were readily mineralized when added to this soil at a concentration of 1 mg/kg (data not shown). The lag between atrazine removal and production of $^{14}CO_2$, and sigmoidal kinetics of $^{14}CO_2$ accumulation indicates growth during the experiment of microorganisms degrading the product(s) excreted by the *Nocardioides*. Overall, these results indicate that atrazine can be efficiently mineralized in this soil through a series of reactions carried out by one, or more than one, organism.

The genes *atzA* and *atzB* were less stable in the *Pseudaminobacter* than in the *Pseudomonas*, so clearly genotypic instability does not account for the relative atrazine-degrading efficacy of these two strains. The rapid onset of atrazine mineralization in the *Pseudaminobacter*-inoculated loam, and the efficacy of both strains in autoclaved soils, indicate that movement of *atzABC* into other bacteria, which could in principle produce atrazine degraders more adapted for this soil, was not a prerequisite for atrazine degradation.

It is commonly hypothesized that chemicals subject to enhanced degradation must be usable as a carbon source (Robertson and Alexander 1994). This study has compared the relative efficacy of bacteria which can and cannot use atrazine as a carbon source for catabolic competence at ecologically reasonable population densities, and the results are in agreement with this hypothesis.

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