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The *atzABC* Genes Encoding Atrazine Catabolism Are Located on a Self-Transmissible Plasmid in *Pseudomonas* sp. Strain ADP†

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***Pseudomonas* sp. strain ADP initiates atrazine catabolism via three enzymatic steps, encoded by *atzA*, *-B*, and *-C*, which yield cyanuric acid, a nitrogen source for many bacteria. In-well lysis, Southern hybridization, and plasmid transfer studies indicated that the *atzA*, *-B*, and *-C* genes are localized on a 96-kb self-transmissible plasmid, pADP-1, in *Pseudomonas* sp. strain ADP. High-performance liquid chromatography analyses showed that cyanuric acid degradation was not encoded by pADP-1. pADP-1 was transferred to *Escherichia coli* strains at a frequency of 4.7×10^{-2}. This suggests a potential molecular mechanism for the dispersion of the *atzABC* genes to other soil bacteria.**

Due to its widespread use over the last 30 years, for both selective and nonselective weed control (2, 39), atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine)] and other *s*-triazine derivatives have been detected in groundwater and surface water at levels exceeding the Environmental Protection Agency's maximum contaminant level of 3 ppb (29).

For the past 3 decades, attempts at isolating bacteria (15, 18) or fungi (28) that mineralize atrazine have been unsuccessful. In the last several years, however, a number of laboratories have independently isolated atrazine-degrading bacteria from sites that were previously exposed to atrazine (1, 4, 5, 10, 31, 32, 35). Our laboratory has studied the genes and enzymes involved in atrazine degradation by *Pseudomonas* sp. strain ADP, in which the first three enzymatic steps are now well defined (3, 11, 13, 37) (Fig. 1). The three genes that encode the enzymes AtzA, -B, and -C have been cloned and sequenced. The first enzyme, AtzA, catalyzes the hydrolytic dechlorination of atrazine, yielding hydroxyatrazine (11). The second enzyme, AtzB, catalyzes hydroxyatrazine deamidation, yielding *N*-isopropylammelide (3). The third enzyme, AtzC (or *N*-isopropylammelide isopropylaminohydrolase), transforms *N*-isopropylammelide to cyanuric acid and isopropylamine (37). An analogous catabolic pathway in *Klebsiella pneumoniae* 99 (25) has been reported to metabolize *s*-triazine compounds, but not atrazine. In that strain, the *trzC*, *-D*, and *-E* genes encode ammelide aminohydrolase, cyanuric acid aminohydrolase, and biuret aminohydrolase, respectively, and are located on a 113-kb plasmid (27).

Several studies have conclusively shown that horizontal gene transfer occurs among microorganisms in natural and laboratory environments (30). The majority of "natural" conjugal transfer experiments have been carried out in nonsterile soil and involve the use of highly promiscuous plasmids and introduced organisms (30, 42). In general, these results suggest that plasmid transfer is a prominent factor in gene flow in natural systems. Previously, horizontal gene transfer has been invoked to explain the appearance of similar *tfd* genes in different 2,4-dichlorophenoxyacetic acid-degrading bacterial strains isolated from different regions (16, 26). Catabolic plasmids have also been implicated in the dispersal of genes for 3-chlorobenzoate, chlorocatechol, and naphthalene biodegradation (17, 22, 34).

Recently, the PCR technique was used to demonstrate the presence of DNA that is strikingly homologous to the *atzABC* genes in atrazine-degrading strains obtained from geographically diverse locations (12). In this study, we report the physical linkage of the *atzA*, *-B*, and *-C* genes on a large plasmid, pADP-1, which is self-transmissible to gram-negative bacteria.

Instability of atrazine degradation phenotype. *Pseudomonas* sp. strain ADP has an unstable atrazine-clearing phenotype during cultivation and propagation on complex laboratory growth media. This phenotypic instability, assayed with the plate-clearing procedure, was especially conspicuous in cells grown with NH_4Cl in the absence of atrazine as the sole nitrogen source (9). Upon repeated subculturing, clearing-negative strains of ADP (Atr^-) that had spontaneously lost the ability to degrade atrazine and hydroxyatrazine on Luria-Bertani (LB) medium (38) containing atrazine or hydroxyatrazine (500 μg/ml) were obtained. These observations suggested that the Atr^- strains were lacking at least the first two enzymes in the atrazine degradation pathway. PCR analyses done with genomic DNA from the Atr^- strains and the *atzA*, *-B*, and *-C* primers confirmed that the first three genes were missing in the Atr^- strains. Moreover, Southern hybridization experiments done with radiolabeled probes specific for the *atzA*, *-B*, and *-C* genes and total genomic DNA isolated from wild-type *Pseudomonas* strain ADP (Atr^+) and the Atr^- strains confirmed that Atr^- strains did not contain DNA homologous to the *atzA*, *-B*, and *-C* genes (data not shown). Phenotypic instability of biodegradation capacity has been observed in other soil bacteria (33, 40).

Transfer of atrazine degradation ability. Based on the instability observed with the atrazine degradation phenotype in

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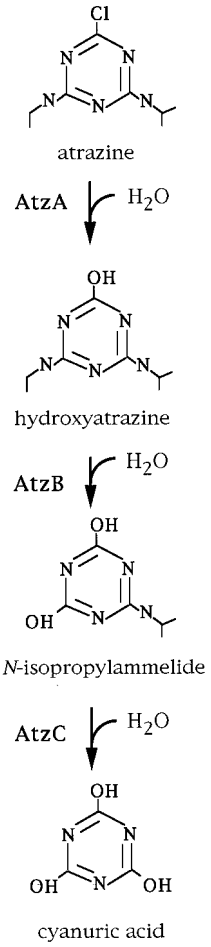

FIG. 1. Pathway for atrazine catabolism to cyanuric acid in *Pseudomonas* sp. strain ADP.

Pseudomonas sp. strain ADP, an experiment was designed to determine if the atrazine degradation genes were located on a plasmid and were self-transmissible. Mating experiments were done in the absence of a helper strain with *Pseudomonas* sp. strain ADP (Nal^r) (Atr^+) as the donor and *Escherichia coli* AD256 (*recA56 srlC300*::Tn*10*; Tet^r) (19) as the recipient. Samples of 2 ml of an overnight culture of *Pseudomonas* sp. strain ADP (donor) and of *E. coli* AD256 (recipient) (19) were centrifuged at 10,000 × *g* for 1 min at 4°C, washed in a solution containing 0.85% sodium chloride and 0.01% Tween 20, and resuspended in 0.1 ml of sterile LB broth. Cell suspensions were placed on LB agar plates and incubated at 30°C overnight. Dilutions of the mating mixtures were plated on LB agar with atrazine (500 μg/ml) and tetracycline (15 μg/ml) and incubated at 37°C overnight.

Fifty *E. coli* colonies (Tet^r) from the mating mixture were analyzed for atrazine degradation ability, and three colonies, designated *E. coli* 3A, 3B, and 3C, degraded atrazine in the plate-clearing assay (13). These colonies did not grow on plates containing nalidixic acid (20 μg/ml), indicating that they were not *Pseudomonas* sp. strain ADP that had acquired resistance to tetracycline. Moreover, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis revealed that the soluble protein profiles of the atrazine-degrading *E. coli* strains were different from those of *Pseudomonas* sp. strain ADP. Mating experiments with *Pseudomonas* sp. strain ADP as the donor strain yielded atrazine-degrading *E. coli* transconjugants at a frequency of 4.7×10^{-2} per recipient. In addition the plasmid encoding atrazine degradation activity was transferable to several gram-negative soil bacteria (6).

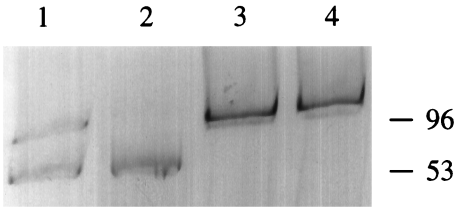

FIG. 2. Plasmid profiles. Lanes: 1, *Pseudomonas* sp. strain ADP (31), 2, Atr^- strain of *Pseudomonas* strain ADP, 3 and 4, transconjugants 3A and 3B from the mating of ADP with *E. coli* strain AD256. The values to the right of the gel are mean molecular masses in kilobases determined with reference to plasmids from *Sinorhizobium* strains (36).

Cell extracts were prepared from *E. coli* 3A, 3B, and 3C, subjected to SDS-polyacrylamide gel electrophoresis with a Mini-PROTEAN II gel apparatus (Bio-Rad), and Western blotted with anti-AtzA antibody (9). These procedures showed that AtzA was expressed in all three transconjugant strains. Cell extracts from wild-type *Pseudomonas* sp. strain ADP served as a positive control. Extracts from *Pseudomonas* sp. strain ADP (Atr^-) and *E. coli* AD256 were used as negative controls, and these strains failed to yield any protein that reacted with the anti-AtzA antibody.

Plasmid content of bacteria. Previously, using conventional plasmid isolation techniques, we failed to observe a plasmid that contained the *atzABC* genes in *Pseudomonas* sp. strain ADP (13). In this study, plasmid profiles were determined on horizontal agarose gels by a modified in-well lysis method (20). Gels were prepared in TBE buffer (89 mM Tris-borate–2 mM EDTA; pH 8.0) with 0.75% (wt/vol) agarose and 1% (wt/vol) SDS. Cells were grown with the appropriate antibiotics as described above. Log-phase cells (400 μl) of *Pseudomonas*, *Sinorhizobium*, or the transconjugant *E. coli* strains were centrifuged, washed with 0.5 M NaCl, resuspended in 50 μl of 20% (wt/vol) sucrose, and added to wells preloaded with modified lysis solution (lysozyme [1.0 mg/ml], RNase [10 μg/ml], and 20% [wt/vol] sucrose in TBE). After 10 min of incubation at room temperature, voltage was applied as follows: 5 V for 30 to 45 min, 14 V for 15 min, 40 V for 60 min, and 80 V for 7 to 8 h. Molecular weights of the *Sinorhizobium* plasmids (21, 36) were used to estimate the sizes of the plasmids present in the *Pseudomonas* and the recombinant *E. coli* strains. The approximate molecular masses in kilobases of the indigenous plasmids in the *Sinorhizobium fredii* strains were as follows: USDA 191, >455, 347, and 105; USDA 205, >455, 342, 177, 126, and 53; USDA 206, >455, 320, 99, and 85; and USDA 217, >455, 335, and 146 (21, 36).

Results of the in-well lysis studies indicated that *Pseudomonas* sp. strain ADP (Atr^+) contained at least two plasmids, pADP-1 and pADP-2, of approximately 96 and 53 kb, respectively (Fig. 2). The pADP-1 plasmid was missing in the Atr^- *Pseudomonas* sp. ADP strains. The *E. coli* transconjugants (Tet^r) that acquired atrazine degradation ability gained a plasmid of approximately the same size (Fig. 2). Plasmid pADP-2 did not hybridize with any of the *atzA*, *-B*, and *-C* gene probes.

Restriction enzyme analyses of plasmid DNA from *E. coli* 3A, 3B, and 3C indicated that all three strains contained identical plasmid bands (data not shown). Total genomic and plasmid DNA was isolated from the *E. coli* clones as described previously (38) and 500 ng of DNA was used as a template in

the PCRs with primers designed specifically for *atzA*, *atzB*, and *atzC* (12, 23). PCR analysis indicated that *atzABC* were present on the plasmid(s) acquired by *E. coli* AD256 (data not shown). Southern blotting and hybridizations were done with radiolabeled probes specific for *atzA*, *-B*, and *-C* genes (12, 38) and plasmid DNA from *E. coli* 3A, 3B, and 3C digested with *Hin*dIII. These data confirmed the PCR results indicating that the *atzABC* genes were located on the transferred plasmid.

In a parallel study, Topp et al. (41) characterized a number of atrazine-degrading bacteria from agricultural soil and found that all the isolates metabolized atrazine through hydroxyatrazine as an intermediate, and one plasmid of approximately 97 kb was common to all the atrazine-catabolizing bacteria. The relationship of this plasmid to pADP-1 remains to be determined.

Presence of atrazine- and cyanuric acid-metabolizing enzymes. To determine if pADP-1 contained a gene that encoded cyanuric acid degradation, resting cells and cell extracts from *Pseudomonas* sp. ADP strains (Atr^+ and Atr^-), *E. coli* 3A (Atr^+), and *E. coli* AD256 (Atr^-) were tested for their abilities to catabolize atrazine and cyanuric acid. High-performance liquid chromatography analysis was done on supernatants from whole resting cells and cell extracts incubated with atrazine or cyanuric acid (200 μg/ml) with a Hewlett-Packard HP 1090 liquid chromatograph system as described previously (11). Atrazine and its metabolites were resolved with a Nova-Pak analytical C_{18} reverse-phase high-performance liquid chromatography column (4-μm spherical packing, 150 by 3.9 mm; Waters Corp.) and an acetonitrile gradient in water at a flow rate of 1.0 ml/min as described previously (11). Cyanuric acid was resolved with an analytical normal-phase column (Lichrosorb RP-18 column, 5-μm spherical packing, 250 by 4.6 mm; Alltech, Deerfield, Ill.) as described previously (37). Authentic atrazine and cyanuric acid were analyzed simultaneously. Cells were incubated with 200 μg of atrazine per ml and 200 μg of cyanuric acid per ml at 30°C for 12 h.

At the end of the experiment, only 16 and 1% of the atrazine with *Pseudomonas* sp. strain ADP (Atr^+) and *E. coli* 3A (Atr^+), respectively, were detectable. However, with *Pseudomonas* sp. strain ADP (Atr^-) and *E. coli* AD256 cells, 80 and 100% of the atrazine, respectively, were recovered. Moreover, while 88 to 98% of the cyanuric acid was recovered after 12 h in cultures of *E. coli* 3A or *E. coli* AD256, with Atr^+ and Atr^- *Pseudomonas* sp. ADP strains, only 30 and 49%, respectively, of the cyanuric acid were detected at the end of the experiment. Similar results were obtained with the cell extracts. These results indicated that the transconjugant *E. coli* strain 3A (Atr^+) acquired only the *atzABC* genes and not genes encoding enzymes involved in the degradation of cyanuric acid. Moreover, *Pseudomonas* sp. strain ADP (Atr^-) that lacked *atzA*, *-B*, and *-C* retained the ability to degrade cyanuric acid. These results suggest that the gene(s) encoding the degradation of cyanuric acid is not located on pADP-1.

In summary, bacterial growth on cyanuric acid is thought to be a relatively common phenotype in soil (7, 8, 14, 24, 25, 43). However, fewer bacteria are thought to catabolize atrazine and these have only been identified recently (1, 4, 5, 10, 31, 32, 35). The *atzABC* genes confer on a host bacterium the ability to metabolize atrazine to cyanuric acid. The identification of the self-transmissible plasmid pADP-1 containing the *atzABC* genes demonstrates a mechanism for conferring atrazine-mineralizing ability on bacteria capable of metabolizing cyanuric acid. In this context, it is important to further delineate the structure and evolution of pADP-1 and related plasmids. Such studies are in progress.

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