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The Atrazine Catabolism Genes *atzABC* Are Widespread and Highly Conserved

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***Pseudomonas* strain ADP metabolizes the herbicide atrazine via three enzymatic steps, encoded by the genes *atzABC*, to yield cyanuric acid, a nitrogen source for many bacteria. Here, we show that five geographically distinct atrazine-degrading bacteria contain genes homologous to *atzA*, *-B*, and *-C*. The sequence identities of the *atz* genes from different atrazine-degrading bacteria were greater than 99% in all pairwise comparisons. This differs from bacterial genes involved in the catabolism of other chlorinated compounds, for which the average sequence identity in pairwise comparisons of the known members of a class ranged from 25 to 56%. Our results indicate that globally distributed atrazine-catabolic genes are highly conserved in diverse genera of bacteria.**

Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine] is a herbicide used for controlling broad-leaf and grassy weeds and is relatively persistent in soils (51). Atrazine and other *s*-triazine compounds have been detected in ground and surface waters at levels exceeding the Environmental Protection Agency's maximum contaminant level of 3 ppb (30).

Microbial populations exposed to synthetic chlorinated compounds, such as atrazine, often respond by producing enzymes that degrade these molecules. Most of our current understanding of the genes and enzymes involved in atrazine degradation derives from studies using *Pseudomonas* strain ADP, in which the first three enzymatic steps in atrazine degradation have been defined (6, 14, 15, 48). The genes *atz A*, *-B*, and *-C*, which encode these enzymes, have been cloned and sequenced. Atrazine chlorohydrolase (AtzA), hydroxyatrazine ethylaminohydrolase (AtzB), and *N*-isopropylammelide isopropylaminohydrolase (AtzC) sequentially convert atrazine to cyanuric acid (6, 14, 15, 48) (Fig. 1). Cyanuric acid and related compounds are catabolized by many soil bacteria (10, 11, 17, 24, 26, 61), and by *Pseudomonas* sp. ADP, to carbon dioxide and ammonia (35). This provides the evolutionary pressure for the *atzA*, *-B*, and *-C* genes to permit bacterial growth on the more than one billion pounds of atrazine that have been applied to soils globally (20). Here we used a knowledge of the *atzA*, *-B*, and *-C* gene sequences to investigate the presence of homologous genes in other atrazine-degrading bacteria. In this study, we report that five atrazine-degrading microorganisms, which were recently isolated from geographically separated sites exposed to atrazine, contained nearly identical *atzA*, *-B*, and *-C* genes.

Atrazine-catabolizing bacteria used in this study. Until recently, attempts at isolating bacteria (18) or fungi (27) that completely degrade atrazine to carbon dioxide, ammonia, and chloride were unsuccessful. While several microorganisms were shown to dealkylate atrazine, they were unable to displace the chlorine atom (41, 54). Since 1994, several research groups have independently isolated atrazine-degrading bacteria that displaced the chlorine atom and mineralized atrazine (3, 7, 13, 35, 39, 46). Six of these bacterial cultures, listed in Table 1, were studied here, and the *Clavibacter* strain had been investigated previously (13).

Detection of *atzA*, *-B*, and *-C* homologs in atrazine-degrading microorganisms by PCR analysis. Recently isolated atrazine-degrading bacteria were screened for the presence of DNA homologous to the *Pseudomonas* strain ADP *atzABC* genes, which encode enzymes transforming atrazine to cyanuric acid (Fig. 1). Total genomic DNA was isolated from each of these bacteria as described elsewhere (49), and the PCR technique was used to amplify sequences internal to the *atzA*, *-B*, and *-C* genes as described elsewhere (13). Custom primers were designed specifically for *atzA* (5′CCATGTGAACCAGA TCCT3′ and 5′TGAAGCGTCCACATTACC3′), *atzB* (5′TC ACCGGGGATGTCGCGGGC3′ and 5′CTCTCCCGCATG GCATCGGG3′), and *atzC* (5′GCTCACATGCAGGTACTC

TABLE 1. Recently isolated atrazine-catabolizing bacteria

Genus	Strain	Location where isolated	Yr reported (reference)
Pseudomonas[a]	ADP	Agricultural-chemical dealership site, Little Falls, Minn.	1995 (35)
Ralstonia[a]	M91-3	Agricultural soil, Ohio	1995 (46, 55)
Mixed culture		Basel, Switzerland	1995 (57)
Clavibacter		Agricultural soil, Riverside, Calif.	1996 (13)
Agrobacterium	J14a	Agricultural soil, Nebraska	1996 (39)
ND[b]	38/38	Atrazine-contaminated soil, Indiana	1996 (3)
Alcaligenes[a]	SG1	Industrial settling pond, San Gabriel, La.	1997 (7)

[a] Isolate identity based on 16S rRNA sequence analysis.
[b] ND, not determined.

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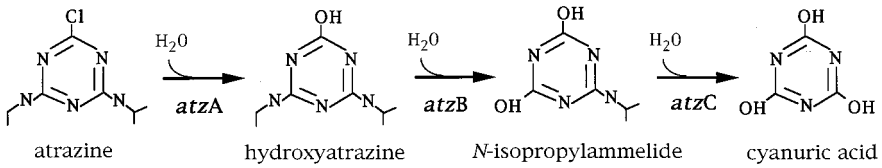

FIG. 1. Pathway for atrazine catabolism to cyanuric acid in *Pseudomonas* sp. strain ADP.

CA3′ and 5′GTACCATATCACCGTTTGCCA3′) by using the Primer Designer package, version 2.01 (Scientific and Educational Software, State Line, Pa.), and were synthesized by Gibco BRL (Gaithersburg, Md.). PCR fragments were amplified by using *Taq* DNA polymerase (Gibco BRL) (22) and were separated from primers on a 1.0% agarose gel. The results of these studies (Fig. 2) indicated that PCR amplification consistently produced DNA fragments of 0.5 kb for all organisms when the *atzA* or *-B* primers were used and fragments of 0.6 kb when the *atzC* primers were used.

Southern hybridization analyses were performed on the PCR-amplified DNA as described elsewhere (49) to confirm the presence of homologous DNA. We used a 0.6-kb *Apa*I/*Pst*I fragment from pMD4 (15), a 1.5-kb *Bgl*II fragment from pATZB-2 (6), and a 2.0-kb *Eco*RI/*Ava*I fragment from pTD2.5 (48) as probes for *atzA*, *-B*, and *-C* genes, respectively. DNA probes were labeled with [α-^{32}P]dCTP by using the Rediprime Random Primer Labeling Kit (Amersham Life Science, Arlington Heights, Ill.) according to the manufacturer's instructions. Southern hybridization analyses, performed under stringent conditions, confirmed that each strain contained DNA homologous to *atzA*, *-B*, and *-C* (data not shown). With strain M91-3 and isolate 38/38, however, in addition to the expected 0.5-kb *atzB* PCR product (Fig. 2, lanes 8 and 10), a 1.2-kb fragment was also obtained. However, no hybridization to this fragment was seen with the *atzB* probe. Similar investigations showed that a mixed culture obtained from Switzerland (Table 1), capable of degrading atrazine, also contained DNA homologous to all three *atz* genes (12).

As a negative control, bacteria known not to degrade atrazine were analyzed. PCR analyses were carried out with genomic DNA from the following randomly chosen laboratory strains: *Rhodococcus chlorophenolicus* (1), *Flavobacterium* sp. (47), *Streptomyces coelicolor* M145 (21), *Amycolatopsis mediterranei* (19), *Agrobacterium* strain A136 and strain A348 (A136/pTiA6NC) (60), *Arthrobacter globiformis* MN1 (45), *Bradyrhizobium japonicum* (33), *Rhizobium* sp. strain NGR 234 (44), *Pseudomonas* NRRLB12228, and *Klebsiella pneumoniae* 99 (16). None of these strains contained DNA that was amplified by PCR using the primers designed to identify the *atzA*, *-B*, or *-C* gene (data not shown).

DNA sequences of *atzA*, *-B*, and *-C* homologs in atrazine-degrading microorganisms. DNAs amplified from the five strains in Table 1 with the *atzA*, *-B*, and *-C* primers were purified from gel slices by using the GeneClean II System (Bio 101, Inc., Vista, Calif.) and sequenced with a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer Corp., Norwalk, Conn.) and an ABI model 373A DNA sequencer (Applied Biosystems, Foster City, Calif.). The GCG sequence analysis software package (Genetics Computer Group, Inc., Madison, Wis.) was used for all DNA and protein sequence comparisons and alignments. Table 2 summarizes these data. The PCR-amplified genes were ≥99% identical to the *Pseudomonas* strain ADP *atzA*, *-B*, and *-C* genes in all pairwise comparisons of DNA sequences. This remarkable sequence identity suggested that each *atz* gene in the different genera was derived from a common ancestor and that they have diverged evolutionarily only to a limited extent.

A review of the literature on other bacterial catabolic pathways indicated a much greater degree of divergence when genes encoding enzymes for the catabolism of other commercially relevant chlorinated compounds were compared (Table 3). As with atrazine, multiple bacterial strains that catabolize 1,2-dichloroethane, chloroacetic acid, 2,4-dichlorophenoxyacetate, dichloromethane, and 4-chlorobenzoate have been isolated. A comparison of the gene sequences encoding the initiating reactions in the catabolism of each of those compounds revealed that sequence divergence was comparatively high. In pairwise comparisons within each gene class, the average sequence identities ranged from 25 to 56% (divergence was 46 to 75%). With the *atzABC* genes, by contrast, there is at most a 1% sequence difference within the sequenced gene region (Table 2). Moreover, the *atzB* sequences were completely identical, and the *atzC* genes diverged by only 1 bp in one of the five strains tested. This suggests that the *atz* genes recently arose from a single origin and have become distributed globally. Similarly, identical parathion hydrolase genes were isolated from two bacteria representing different genera and global locations (40, 52, 53).

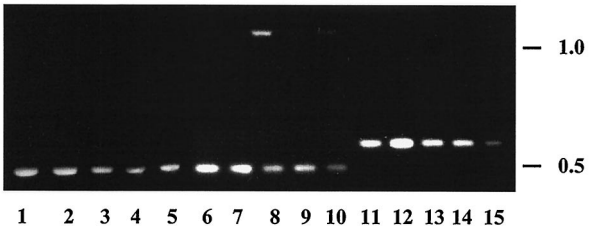

FIG. 2. PCR analysis with primers designed to amplify internal regions of *atzA* (lanes 1 to 5), *atzB* (lanes 6 to 10), and *atzC* (lanes 11 to 15). The atrazine-degrading bacteria analyzed were *Pseudomonas* strain ADP (35) (lanes 1, 6, and 11), *Alcaligenes* strain SGI (7) (lanes 2, 7, and 12), *Ralstonia* strain M91-3 (46) (lanes 3, 8, and 13), *Agrobacterium* strain J14a (39) (lanes 4, 9, and 14), and isolate 38/38 (3) (lanes 5, 10, and 15). Values to the right of the gel are sizes (in kilobase pairs).

TABLE 2. Sequence identities of *atzABC* homologs from different atrazine-degrading bacteria

Strain	% DNA sequence identity[a]		
	atzA	*atzB*	*atzC*
Pseudomonas ADP	100	100	100
Alcaligenes SG1	99.2	100	100
Ralstonia M91-3	99.0	100	100
Agrobacterium J14a	99.1	100	100
Isolate 38/38	99.3	100	99.8

[a] DNA sequences obtained from each strain by using the *ataA*, *-B*, and *-C* primers were compared with the *atzABC* gene sequences from *Pseudomonas* strain ADP.

TABLE 3. Sequence comparisons of isofunctional bacterial enzymes that catabolize chlorinated compounds

Gene	Enzyme	Average % protein sequence identity[a] (no. of pairwise comparisons)	References
dhlA, *dhaA*	Haloalkane dehalogenase	25.0 (1)	23, 31
dehC, *hadL*, *dehH*, *dehH1*, *dehH2*, *dhlB*, *dehCI*, *dehCII*	2-Haloacid dehalogenase	36.6 ± 3.9 (36)	5, 25, 28, 29, 42, 43, 50, 59
tfdA	2,4-Dichlorophenoxyacetate monooxygenase	43.2 ± 4.6 (21)[b]	34, 37, 38, 56, 58
dcmA	Dichloromethane dehalogenase	56.0 (1)	4, 32
atzA	Atrazine chlorohydrolase	98.6 ± 0.12 (15)[c]	This study
atzB	Hydroxyatrazine ethylaminohydrolase	100 (10)[c]	This study
atzC	*N*-Isopropylammelide isopropylaminohydrolase	99.0 ± 0.43 (10)[c]	This study

[a] All possible pairwise alignments of translated gene sequences were made. The average percent identity is the mean of the percent identity values for all pairwise alignments ± standard error of the mean.

[b] Includes full protein sequences as well as partial protein sequences of ≥100 amino acids.

[c] Sequence identity within a 0.5-kb PCR product for *atzA* and *-B* and within a 0.6-kb PCR product for *atzC*. Six sequences were analyzed for *atzA*, and five were analyzed for *atzB* and *-C*.

The data presented here provide further support for previous studies suggesting that hydroxyatrazine in the environment derives from biological processes (36), and not solely from abiotic reactions (2, 9). The present data, and a recent report by Bouquard et al. (8), indicate that the gene encoding atrazine chlorohydrolase is widespread in the United States and Europe.

Our observations argue for a single, recent evolutionary origin of the *atz* genes and their subsequent global distribution. We have recently localized the *atzA*, *-B*, and *-C* genes to a large, self-transmissible plasmid in *Pseudomonas* strain ADP (12), and possible mechanisms of transfer of the *atzABC* genes are currently under investigation.

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