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Complete Nucleotide Sequence and Organization of the Atrazine Catabolic Plasmid pADP-1 from *Pseudomonas* sp. Strain ADP

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The complete 108,845-nucleotide sequence of catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP was determined. Plasmid pADP-1 was previously shown to encode AtzA, AtzB, and AtzC, which catalyze the sequential hydrolytic removal of s-triazine ring substituents from the herbicide atrazine to yield cyanuric acid. Computational analyses indicated that pADP-1 encodes 104 putative open reading frames (ORFs), which are predicted to function in catabolism, transposition, and plasmid maintenance, transfer, and replication. Regions encoding transfer and replication functions of pADP-1 had 80 to 100% amino acid sequence identity to pR751, an IncP plasmid previously isolated from *Enterobacter aerogenes*. pADP-1 was shown to contain a functional mercury resistance operon with 99% identity to Tn5053. Complete copies of transposases with 99% amino acid sequence identity to TnpA from IS1071 and TnpA from *Pseudomonas pseudocaligenes* were identified and flank each of the *atzA*, *atzB*, and *atzC* genes, forming structures resembling nested catabolic transposons. Functional analyses identified three new catabolic genes, *atzD*, *atzE*, and *atzF*, which participate in atrazine catabolism. Crude extracts from *Escherichia coli* expressing AtzD hydrolyzed cyanuric acid to biuret. AtzD showed 58% amino acid sequence identity to TrzD, a cyanuric acid amidohydrolase, from *Pseudomonas* sp. strain NRRLB-12227. Two other genes encoding the further catabolism of cyanuric acid, *atzE* and *atzF*, reside in a contiguous cluster adjacent to a potential LysR-type transcriptional regulator. *E. coli* strains bearing *atzE* and *atzF* were shown to encode a biuret hydrolase and allophanate hydrolase, respectively. *atzDEF* are cotranscribed. AtzE and AtzF are members of a common amidase protein family. These data reveal the complete structure of a catabolic plasmid and show that the atrazine catabolic genes are dispersed on three disparate regions of the plasmid. These results begin to provide insight into how plasmids are structured, and thus evolve, to encode the catabolism of compounds recently added to the biosphere.

Many bacteria contain plasmids that carry genes functional in antibiotic resistance, virulence for animal or plant hosts, or the catabolism of diverse chemical compounds. While much has been learned since Lederberg’s initial discovery of plasmids in 1952 (36), genomic approaches will further enhance our understanding of plasmid structure and evolution. Recently, the complete nucleotide sequences of approximately 90 bacterial and 10 archeal plasmids have been obtained (5, 20, 29, 41, 46, 52, 59; http://www.ncbi.nlm.nih.gov:80/PMGifs/Genomes/eub_p.html). However, most of the sequenced plasmids are relatively small, are used as vectors in molecular biology, or are of medical importance. The latter group of plasmids consist predominantly of those known to contain antibiotic resistance genes or encode virulence determinants associated with infectious diseases (5, 29, 52). In contrast, catabolic plasmids, which have been identified in many non-pathogenic soil bacteria (49), have been shown to transfer among bacteria, thus disseminating genes encoding the metabolism of environmentally relevant compounds (11). For example, plasmids encoding the catabolism of toluene, camphor, naphthalene, and 2,4-dichlorophenoxyacetate are known (3, 12, 45, 64). Many of the genes involved in these respective catabolic pathways have been cloned and sequenced, but to date only one catabolic plasmid, pNL1, from *Sphingomonas aromaticivorans* strain F199 has been completely sequenced (46). This plasmid contains genes encoding enzymes for the metabolism of biphenyl, naphthalene, *m*-xylene, and *p*- cresol.

Metabolism of the herbicide atrazine has also been shown to be linked to catabolic plasmids. Mandelbaum et al. (38) isolated *Pseudomonas* sp. strain ADP, which metabolizes atrazine to carbon dioxide and ammonia. The first three enzymatic steps, encoded by the genes *atzA*, *atzB*, and *atzC*, transform atrazine to cyanuric acid (9, 15, 47). Cyanuric acid has been shown to be a common intermediate in the degradation pathways for melamine (2,4,6-triamino-s-triazine) and atrazine (21, 22, 47) in *Pseudomonas* sp. strain NRRLB-12227 and *Pseudomonas* sp. strain ADP, respectively (Fig. 1). However, while the hydrolysis of cyanuric acid in *Pseudomonas* sp. strain NRRLB-12227 proceeds through biuret and urea intermediates, the reactions involved in cyanuric acid degradation in *Pseudomonas* sp. strain ADP were not established.

The *atzA*, *atzB*, and *atzC* genes in *Pseudomonas* sp. strain ADP have been localized to an approximately 100 kb plasmid, pADP-1 (17), and DNA regions with homology to IS1071 have been shown to flank *atzA* (18). Recently, plasmid-localized
genes homologous to the atzA, atzB, and atzC genes have been identified in different genera of atrazine-degrading bacteria isolated from geographically diverse locations (16; B. Martinez, M. de Souza, L. Wackett, and M. Sadowsky, Abstr. 99th Gen. Meet. Am. Soc. Microbiol., abstr. Q352, 1999). Since the atrazine catabolic genes have >99% sequence identity among different species of bacteria isolated independently from different continents, this suggests a recent evolution and dissemination of the atzA, atzB, and atzC genes.

To further our understanding about the assembly of the atrazine catabolic pathway and the accessory functions encoded by pADP-1, we undertook the complete sequencing and annotation of this plasmid. Sequence analysis revealed that the gene regions encoding plasmid replication, transfer, and maintenance functions of pADP-1 were nearly identical to those found on pR751, an IncP plasmid from Enterobacter aerogenes. Plasmid pADP-1 is predicted to encode 104 proteins. A functional mercury resistance operon is also present on pADP-1. Structural and functional studies showed that the genes encoding the initial reactions of atrazine catabolism are not organized in an operon, but are dispersed and flanked by transposases. Moreover, genes for the complete catabolism of cyanuric acid to CO₂ and NH₃ were localized to pADP-1.

MATERIALS AND METHODS

Isolation of pADP-1 DNA. The pADP-1 plasmid was introduced into Escherichia coli AD256 by conjugation as described previously (17). Cells were grown overnight in one-fourth-strength Luria-Bertani (LB) medium (48) containing 500 µg of ampicillin per ml. Cells were harvested by centrifugation at 8,000 × g for 10 min at 4°C. Large-scale plasmid DNA isolation preparations were done as described by Hirsch et al. (27), and pADP-1 was further purified using CsCl buoyant-density ultracentrifugation (48).

Construction of pADP-1 shotgun library. Plasmid pADP-1 DNA was nebulized using 4.4 × 10⁸ Pa of N₂ for 4.0 min and a nebulizer (IPT Medical Products Inc., Chicago, Ill.). The DNA ends were filled in, ligated into plasmid pUC18, and transferred to DH10B (Gibco-BRL, Grand Island, N.Y.) as described previously (48). Clones containing inserts were picked randomly using the Genetix Q-Bot robot (Genetix Ltd., New Milton, United Kingdom) and stored in 96-well microtiter plates.

DNA sequencing. DNA templates from randomly selected shotgun clones were prepared from 3-ml overnight cultures grown in LB medium containing 50 µg of ampicillin per ml using an AutoGene 740 DNA isolation system (Integrated Separation Systems, Framingham, Mass.). DNA from each preparation was dissolved in 80 µl of sterile H₂O and stored at ∼20°C until used. Plasmid DNA, 500 ng, was sequenced using M13 forward and reverse primers and an ABI BigDye cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and an ABI model 377 DNA sequencer (Applied Biosystems).

Sequence assembly. The Phred/Phrap/Consed sequence analysis software package was used to cross-match vector sequences and assemble the pADP-1 sequence into contigs (25). For gap closure, custom primers were designed from the ends of each contig in the pADP-1 sequence using Primer Designer software, version 2.01 (Scientific and Educational Software, State Line, Pa.). Primers were synthesized by Integrated DNA Technologies, Inc (Coralville, Iowa). DNA fragments containing sequences necessary to close gaps between contigs were obtained by using the PCR high-fidelity Tth polymerase and purified plasmid pADP-1 DNA as a template. PCR was done using a Perkin-Elmer/Applied Biosystems XL Polymerase DNA amplification kit (PE/Applied Biosystems, Foster City, Calif.) and a PTC-100 thermocycler (MJ Research, Inc., Nepean, Ont.). PCR products were purified using a Qiagen gel extraction kit (Qiagen, La Jolla, Calif.) and sequenced directly as described above. The pBluescript vector (Stratagene) was used to clone PCR products longer than 3 kb and facilitate sequencing.

Analysis of ORFs. The analysis of the open reading frames (ORFs) present in the pADP-1 sequence was completed using the web-based versions of GeneMark (7; http://genemark.biology.gatech.edu/GeneMark/), GeneMark.hmm (37; http://genemark.biology.gatech.edu/GeneMark/hmmchoice.html), Ptam (http://ptam.wustl.edu/), and the NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) programs. ORFs that were consistently predicted by these programs were selected and met the following criteria: (i) the start codon was ATG, GTG, or TTG; (ii) the stop codon was TAG, TAA, or TGA; and (iii) the size of the ORF was between 150 and 5,000 bp. Start codon positions were assigned using manual identification of the Shine-Dalgarno sites within 15 bp of a potential start site. GeneMark.hmm (37) was also used to examine potential frameshifts leading to misidentification of ORFs. Those ORFs predicted to encode genes were analyzed further to determine their homology to proteins of known function using BLAST and BLASTP (http://seqsim.ncgr.org/newBlast.html).

Cloning of the cyanuric acid hydrolase gene. The putative cyanuric acid hydrolase gene, atzD, was cloned from pADP-1 using a PCR approach and primers CAHFF (5'-GCGATCCTGCGTTCATCGACAGAG-3') and CAHFR (5'-GCGATCCAGATGGGCTGATGCT-3'). The primers contained BamHI restriction sites at each end to facilitate gene cloning. PCR was performed using a high-fidelity XL polymerase DNA amplification kit (PE/Applied Biosystems). Amplification of the 1.4-kb DNA fragment was achieved using the following conditions: 94°C for 1 min, and then 28 cycles consisting of 94°C for 15 s and 60°C for 1 min. The PCR product was resolved on a 0.8% agarose gel, and the band was excised from the gel and purified using the Qiagen gel extraction kit (Qiagen, La Jolla, Calif.). The PCR product was cloned into the BamHI site of pK transformants (4) using standard cloning procedures (48). Potential clones were screened for the insert by restriction enzyme digestions using BamHI, and one clone, pBMZ1, was used for subsequent functional analyses.

Cyanuric acid degradation assays. To determine if the putative atzD gene encoded a cyanuric acid amidohydrolase, the hydrolysis of cyanuric acid by crude extracts of E. coli DH5α(pBMZ1) and E. coli DH5α was examined. Crude extracts were prepared as described previously (9). Reaction mixtures contained 10 mM potassium phosphate buffer (pH 7.2), 3 mM cyanuric acid (Sigma-Aldrich, St. Louis, Mo.), and 100 µg of protein in a final reaction volume of 1.0
ml. Replicate reactions were incubated at 30°C and at various times terminated by heating at 95°C for 2 min. Samples were centrifuged at 14,000 × g for 10 min, filtered through 0.2-μm filters, and placed in vials for analysis by high-performance liquid chromatography (HPLC). The disappearance of cyanuric acid was monitored by HPLC analysis using an analytical Absorbosphere C18 reverse-phase HPLC column (5-μm spherical packing; 250 by 4.6 mm) (Alltech Associates, Deerfield, Ill.). The isocratic mobile phase was 5 mM potassium phosphate buffer (pH 6.8) containing 5 mM dodecyltritylmethylammonium phosphate and Q12 ion pair cocktail (Regis Chemical Technologies, Morton Grove, Ill.) at a flow rate of 1.0 ml per min. Spectral data of the column eluent were acquired at 224 nm. Under these conditions, cyanuric acid eluted from the column at about 6.0 min and biuret at 3.3 min. The concentration of cyanuric acid in the sample analyzed was obtained by integrating peak areas at 200 nm.

Waters Corp., Milford, Mass.). The isocratic mobile phase consisted of 5 mM potassium phosphate buffer (pH 7.0), 0.6 mM biuret, and cell extract (1 mg of protein), in a final volume of 10 ml, were incubated at 30°C. Reactions were terminated by heating at 95°C for 2 min. Samples were centrifuged at 14,000 × g for up to 1 week. The appearance of clearing zones surrounding cell growth indicated biuret degradation.

Ammonia release assays were done in 50 mM sodium phosphate buffer (pH 8.0) containing 3 mM biuret and cell extract (1 mg of protein) from E. coli (p11A07). Reaction mixtures were incubated at 30°C for 6 h. Samples were taken at several time points, and the reactions were stopped by addition of 1 N H2SO4. For urea-coupled assays, reaction mixtures were treated with 200 μl of solution A (0.5% NaOH and 0.84% sodium hypochlorite in water). Reactions were incubated at room temperature for 1 h, and the resulting indophenol was monitored at 630 nm.

The disappearance of biuret was also examined by HPLC analyses. Extracts of E. coli DH5α(p11A07) and E. coli DH5α(pUC18) were prepared as described previously (9). Reaction mixtures containing 50 mM sodium phosphate buffer (pH 7.0), 0.6 mM biuret, and cell extract (1 mg of protein), in a final volume of 10 ml, were incubated at 30°C. Reactions were terminated by heating at 95°C for 3 min. Samples were centrifuged at 14,000 × g for 10 min, filtered through 0.2-μm filters, and analyzed by HPLC. The disappearance of biuret was followed by using a Waters System Module A HC anion-exchange column (150 mm by 4.6 mm; Waters Corp., Milford, Mass.). The isocratic mobile phase consisted of 5 mM sodium phosphate buffer (pH 7.0) at a flow rate of 0.5 ml per min (35). Spectral data of the column eluent were collected at 224 nm. Under these conditions, cyanuric acid eluted from the column at about 6.0 min and biuret at 3.3 min. The concentration of cyanuric acid in the sample analyzed was obtained by integrating peak areas at 200 nm.

Ammonia release assays. Plate-clearing, ammonia release, thin-layer chromatography (TLC), and HPLC assays were used to determine if ORF101 (atzE) encoded biuret hydrolase activity. One clone from the pADP-1 sequencing library, p11A07, containing a complete copy of ORF101 was used for these analyses. For the plate-clearing assays, 500 μl of an E. coli(p11A07) culture (optical density at 600 nm [OD600] = 1.0) was plated onto the surface of one-fourth-strength LB medium plates containing 60 mM sodium phosphate buffer and 1,000 μg of biuret per ml. Plates were incubated at 37°C for up to 1 week. The appearance of clearing zones surrounding cell growth indicated biuret degradation.

Ammonia release assays were done in 50 mM sodium phosphate buffer (pH 8.0) containing 3 mM biuret and cell extract (1 mg of protein) from E. coli (p11A07). Reaction mixtures were incubated at 30°C for 6 h. Samples were taken at several time points, and the reactions were stopped by addition of 1 N H2SO4. For urea-coupled assays, reaction mixtures were treated with 200 μl of solution A (0.5% NaOH and 0.84% sodium hypochlorite in water). Reactions were incubated at room temperature for 1 h, and the resulting indophenol was monitored at 630 nm.

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The cultures were incubated for up to 1 week at 37°C, and urea hydrolysis was monitored by observing a change in the color of the medium, indicating an increase in pH due to urea hydrolysis. Ammonia release assays were done to determine if urea or allophanate was a substrate for ORF101. These data were done using an analytical Absorbosphere C18 reverse-phase HPLC column (5-μm spherical packing; 250 by 4.6 mm) (Alltech Associates, Deerfield, Ill.). The isocratic mobile phase was 5 mM potassium phosphate buffer (pH 6.8) containing 5 mM dodecyltritylmethylammonium phosphate and Q12 ion pair cocktail (Regis Chemical Technologies, Morton Grove, Ill.) at a flow rate of 1.0 ml per min. Spectral data of the column eluent were acquired at 224 nm. Under these conditions, cyanuric acid eluted from the column at about 6.0 min and biuret at 3.3 min. The concentration of cyanuric acid in the sample analyzed was obtained by integrating peak areas at 200 nm.
RESULTS

Nucleotide sequence, physical map, and genetic organization of pADP-1. The atrazine catabolic plasmid pADP-1 is 108,845 bp, with an overall G+C content of 62.6 mol%. This value is within the range of those found in several genera of gram-negative soil bacteria, including *Pseudomonas* and *Alcaligenes* strains (28, 40). A circular physical map of pADP-1 is shown in Fig. 2. The assembly of pADP-1 was verified by using restriction enzyme analyses and PCR (data not shown). This plasmid contains a classical IncPβ backbone that consists of two regions involved in plasmid conjugation (the *tra* and *trb* operons), an origin of replication (*oriV*), and a region involved in plasmid control, stable inheritance, and partitioning. The nucleotide sequence of the pADP-1 backbone is 80 to 100% identical to the backbone of the archetype IncPβ plasmid R751 except for *kleG*. The main catabolic region of pADP-1 lies outside the *trb* and *tra* operons. This region contains the first three genes necessary for the degradation of atrazine, *atzA*, *atzB*, and *atzC*. In addition, the region contains a mercury resistance operon and several insertion sequence elements. Restriction enzyme analysis of pADP-1 showed that the catabolic region of this plasmid contains numerous restriction sites, while only a few enzymes cut the backbone.

Analyses of ORFs. A total of 104 ORFs were identified in the pADP-1 sequence using GeneMark, GeneMark.hmm, and NCBI ORF Finder (Fig. 3). All three ORF-finding programs identified the same ORFs. Based on significant similarities to proteins of known function, putative functions were assigned to a majority of the ORFs (Table 1); 15% of the ORFs were predicted to be involved in catabolism, 15% were predicted to be involved in transposition, 5% encode putative transporters, 3% are putative transcriptional regulators, 3% are proteins involved in general metabolism, and 39% are proteins necessary for plasmid maintenance, transfer, and replication. Several ORFs (8%) had >38% amino acid identity to hypothetical proteins, and 2% had no significant homology to any known proteins in databases. The first ORF after the origin of replication, *oriV*, was arbitrarily assigned as ORF1 (Table 1 and Fig. 3). More detailed descriptions of many of the ORFs and genes on pADP-1 are found below.

Similarities and differences between pADP-1 and pR751. Sequence analyses revealed that the regions of pADP-1 containing replication, basic transfer, and maintenance functions were nearly identical to gene regions encoding these functions in the IncPβ plasmid pR751 from *Enterobacter aerogenes*. The *tra* and *trb* operons of pADP-1 and pR751 contain genes nec-
necessary for plasmid transfer and replication (59). In addition, the origin of replication and the IncC, KorA, KorB, and Klc proteins, which are involved in the regulation of replication and maintenance of pR751, are highly conserved in pADP-1 (Table 1). These observations suggest that pADP-1 and pR751 have a common ancestor.

Despite having similarity in backbone structure, the regions between oriV and trfA of plasmids pR751 and pADP-1 are different. In pR751, two transposons, Tn4221 and Tn402/Tn5090, are located in the regions between oriV-trfA and trb-tra, respectively; these transposons are not present in pADP-1. Moreover, in pADP-1 two complete copies of a transposase similar to TnpA from IS801 flank the region between trb and tra. In pADP-1, this 52-kb region resembled a

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**Symbols:**
- **tra genes**
- **atrazine catabolic genes**
- **IS1071**, **IS801**-like transposase genes
- **mer**- mercury resistance genes
- **pdhL** and **pdhB** homologs
- Potential regulatory genes
- **trb** genes
- **genes involved in plasmid replication and maintenance.**

**FIG. 3:** Linear map showing graphical representation of ORFs present on the pADP-1 sequence. Boxes above the lines refer to ORFs on the top strand, while those below the line are from the complementary strand. Genes of the same type (operons, insertion sequence [IS] elements, and catabolic genes) have similar shading, and ORFs with putative functions are listed by their numbers. ORFs and genes have been numbered relative to the origin of replication (oriV). Distance between tick marks is 1.3 kb.
<table>
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<tr>
<th>ORF no.</th>
<th>Positions (bp)*</th>
<th>Function of closest homology to others</th>
<th>Source microorganism</th>
<th>No. of amino acids, pADP-1/relative</th>
<th>% Amino acid identity*</th>
<th>GenBank accession no.</th>
<th>E value</th>
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<td>Relaxosome stabilization protein (TraH)</td>
<td>Enterobacter aerogenes pR751</td>
<td>130,130 100 (130/130)</td>
<td>AAC64476.1 1.00E–73</td>
<td></td>
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<tr>
<td>23</td>
<td>14780–16693 d</td>
<td>DNA transport protein (TraG)</td>
<td>Enterobacter aerogenes pR751</td>
<td>637,637 100 (636/637)</td>
<td>AAC64474.1 0.00E</td>
<td></td>
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<tr>
<td>24</td>
<td>16690–17226 d</td>
<td>Maturation peptidase (TraF)</td>
<td>Enterobacter aerogenes pR751</td>
<td>176,178 100 (178/178)</td>
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<tr>
<td>25</td>
<td>17238–19332 c</td>
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<td>687,687 85 (593/687)</td>
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<td>19323–19712 d</td>
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<td>AAC64468.1 0.00E</td>
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<tr>
<td>28</td>
<td>24327–25175 c</td>
<td>Cointegrate formation and resolution protein (IstB)</td>
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<td>262,264 69 (170/245)</td>
<td>BAA39790.1 1.00E–90</td>
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**TABLE 1. Localization and predicted functions of ORFs in pADP-1**

Continued on following page
nested transposon structure and contains all of the genes necessary for the degradation of atrazine to cyanuric acid, a complete mercury resistance operon, and several copies of different transposable elements.

Some of the inverted repeats postulated to be involved in the acquisition of foreign DNA in pR751 are also present in pADP-1 (59). All the inverted repeats in the area between orfV and trfA on pR751 except for inverted repeat 4, are present and highly conserved in pADP-1. In addition, the inverted repeats in pR751 between upf54.4 (traO) and traM are present and highly conserved in pADP-1. However, the inverted repeats present in the areas between Tra1-Tn402 and Tn402-Tra2 in pR751 were not found in the pADP-1 genome.

### Transposition and Integration-related ORFs

About 15% of the identified ORFs in the pADP-1 sequence had significant identity to proteins involved in DNA integration and transposition. Three ORFs (ORF34, ORF41, and ORF49) in the pADP-1 sequence had 99% amino acid sequence identity to TnpA from IS1071. Two IS1071 insertion elements flank the atrazine chlorohydrolase gene, atzA (ORF37), while the third copy is located approximately 6 kb downstream of the hydroxyatrazine ethylaminohydrolase (atzB) gene (ORF44). A truncated copy of transposase TnpA from IS1071 was localized at nucleotide positions 75908 to 76264 near the trb operon (Fig. 3). The 110-bp inverted repeats normally associated with IS1071 are present in the homologs found on pADP-1. In addition, a partial copy (69 nucleotides) of these inverted repeats was found between the istB and the traC genes. DNA fragments homologous to the pdhL and pdhB genes were found to flank most of the copies of IS1071 present on pADP-1. These genes encode different components of the pyruvate dehydrogenase enzyme complex (26). The IS1071 copies present in pADP-1 (Table 1) always interrupt pdhL at the same position, suggesting that pdhL is a target for the insertion of this transposon. Four ORFs, ORF32 (nucleotides 27514 to 29061), ORF39 (37037 to 38584), ORF47 (48776 to 50323), and ORF73 (72897 to 74444), had more than 99% amino acid sequence identity to an...
FIG. 4. (A) Degradation of cyanuric acid by crude cell extracts from *E. coli* DH5α(pBMZ1). Plasmid pBMZ1 contains *atzD* from pADP-1 cloned into the BamHI site of pKT230. Symbols: ●, *E. coli* DH5α(pBMZ1); ○, *E. coli* DH5α. (B) Degradation of biuret by crude cell extracts from *E. coli* DH5α (*atzE*). Symbols: ■, *E. coli* DH5α (*atzE*); □, *E. coli* DH5α(pUC18). (C) Degradation of allophanate by crude cell extracts of *E. coli* DH5α (*atzF*). Symbols: ●, *E. coli* DH5α (*atzF*); ○, *E. coli* DH5α(pUC18). Values are the means of three replicates. Bars indicate standard deviations of the mean.
IS01-like transposase (TnpA) previously identified in *Pseudomonas pseudoalcaligenes* JS45 (14). Complete copies of this transposase were identified on the opposite strand flanking the *atzA* and *atzB* genes and on the same strand upstream of the *atzC* gene (ORF71) (Fig. 3). No inverted repeat structures were found in the DNA regions surrounding these transposases. However, 187-bp and 108-bp conserved DNA regions were also found in the same positions in IS801 (accession number AF086815.2), *Pseudomonas huttiensis* (accession number AF028594), and *Pseudomonas pseudoalcaligenes* (14). These conserved regions have 40% identity to each other, do not contain direct or inverted repeats, and have no homology to other well-characterized transposons.

We also identified additional ORFs with homology to transposases from organisms of diverse genera. For example, ORF68 (nucleotides 66429 to 67739) had 45% identity to a putative transposase from *Acetobacter pasteurianus* (58), ORF70 (69053 to 70021) had 66% identity to a transposase from *Ralstonia solanacearum* (Y. A. Lee, unpublished data; GenBank accession no. AAD49338), and ORF92 (90795 to 92165) was 29% identical to a putative transposase from *Streptococcus pyogenes* (6). In addition, our analysis revealed the presence of ORF104 (107219 to 108286), which had 69% sequence identity to an integrase-like protein from Ts5041 in *Pseudomonas* sp. strain KHP41 (33). Two ORFs, ORF28 (24327 to 25175) and ORF29 (25159 to 25416), had homology to the IstB and IstA proteins from *Ralstonia eutropha*, respectively (51). In plasmids carrying the insertion sequence IS21, *istAB* are usually transcribed as part of an operon (51). In pADP-1, the IstB homolog appears complete, while the IstA homolog (ORF29) is truncated.

The complete nucleotide sequence of pADP-1 revealed the presence of several genes involved in catabolism (Table 1 and Fig. 3). These genes and gene families are discussed below in detail.

Atrazine catabolic genes. Sequence analysis showed that three previously identified genes involved in the initial steps of atrazine catabolism, *atzA*, *atzB*, and *atzC* (9, 15, 47), are localized to different regions of pADP-1 and are not organized in an operon-like structure. The first enzyme, *AtzA*, catalyzes the hydrolytic dechlorination of atrazine, yielding hydroxylatrazine. The second enzyme, *AtzB*, catalyzes hydroxylatrazine deamidation, yielding *N*-isopropylamidamide, and the third, *AtzC*, encodes *N*-isopropylamidamide isopropylaminohydrolase activity, stoichiometrically catalyzing *N*-isopropylamidamide to cyanuric acid and *N*-isopropylamine. Consistent with our previously reported cosmid sequencing efforts, the *atzA* and *atzB* genes are located about 8 kb apart on pADP-1 (9). The *atzC* gene is located about 34 and 25 kb from the *atzA* and *atzB* genes, respectively. Sequence analysis confirmed that the G+C content of *atzC* (44%) was lower than those of *atzA* (58%) and *atzB* (61%) and most of the other genes present in the pADP-1 sequence. This result suggests that *atzC* was acquired from an organism with vastly different G+C content (47).

New catabolic genes involved in atrazine catabolism. ORF100 (nucleotides 101053 to 102144) had 58% amino acid identity to TrzD, a cyanuric acid amidohydrolase from *Pseudomonas* sp. strain NRRLB-12227 (30). Since *AtzC* in *Pseudomonas* sp. strain ADP transforms *N*-isopropylamidamide to cyanuric acid, we postulated that cyanuric acid was transformed by a similar enzyme in this bacterium. However, we previously were unsuccessful in using a trzD gene probe to find this gene in *Pseudomonas* sp. strain ADP or to demonstrate transformation of cyanuric acid in *E. coli* AD256(pADP-1) (M. L. de Souza and I. R. Fruchey, personal communication). To determine whether ORF100 functionally encoded cyanuric acid amidohydrolase in *Pseudomonas* sp. strain ADP, PCR was used to clone a 1.4-kb DNA fragment from pADP-1 into the BamHI site of pKT230, resulting in plasmid pBMZ1. Crude extracts of *E. coli* DH5α and *E. coli* DH5α(pBMZ1) were tested for their ability to hydrolyze cyanuric acid, as evidenced by HPLC analysis. Results in Fig. 4A show that crude extracts of *E. coli* (pBMZ1) had the ability to hydrolyze cyanuric acid, whereas the control *E. coli* strain did not transform this substrate. Mass spectrophotometric studies done using crude cell extracts of *E. coli* (pBMZ1) and cyanuric acid as the substrate indicated that biuret is the product of this reaction (I. R. Fruchey, unpublished data). These results indicate that pADP-1 encodes a functional cyanuric acid amidohydrolase that is homologous to TrzD. This enzyme, AtzD, is encoded in the region between *orfIV* and *trfA*. We are currently in the process of purifying and characterizing AtzD, and these results will be presented elsewhere.

Two ORFs, ORF101 and ORF102, were located directly downstream of *atzD*. ORF101 (102427 to 103800) had 37% sequence identity with a nicotinamidase/pyrazinamidase from *Mycobacterium smegmatis* (8) (GenBank accession no. AAC 77368), while ORF102 (104283 to 106100) had 44% sequence identity to urea amidolase from *Saccharomyces cerevisiae* (GenBank accession no. CAAA5172) (24, 57, 63). Multiple sequence alignments revealed that ORF101 and ORF102 had conserved sequence signatures diagnostic of an amidase protein family (Fig. 5) (34, 43). The residues corresponding to Asp184 and Ser188 in ORF102 and Asp169 and Ser173 in ORF101 are conserved in all the members of this amidase family. In addition, the residues corresponding to Lys90, Ser164, Ser165, Ser188, and Arg190 in ORF102 and Lys73, Ser149, Ser150, Ser173, and Arg175 in ORF101 are conserved in all the members of this same amidase family (34, 43). Mutagenesis studies have shown that these five residues are critical for the amidase activity of fatty acid amidohydrolase, a mammalian amidase that hydrolyses fatty acid amides (43). Based on our observations, we propose here that ORF101 and ORF102 are new members of this amidase family.

Growth studies indicated that *Pseudomonas putida* PRS2000 (pADP-1) transconjugants had the ability to use cyanuric acid as the sole nitrogen source (data not shown), while the wild-type strain did not. This result indicated that the gene(s) necessary for further catabolism of cyanuric acid was present on pADP-1. Since biuret is the product of cyanuric acid degradation in *Pseudomonas* sp. strains ADP and NRRLB-12227 and contains multiple amide bonds, the data suggested that ORF101 and ORF102 were involved in biuret metabolism. To experimentally determine whether these ORFs encoded a functional biuret amidohydrolase, *E. coli* (p11A07) and *E. coli* (p14D12), containing ORF101 and ORF102, respectively, were spotted onto the surface of LB agar medium containing 4,000 μg of biuret per ml. While a clear zone surrounding cell
growth of *E. coli* DH5α(p11A07) was found after 7 days of incubation, no such zone was detected with *E. coli*(p14D12) or the *E. coli*(pUC18) negative control (data not shown). To confirm these results, cell extracts from *E. coli* DH5α(p11A07) were examined for biuret hydrolysis using HPLC and ammonia release assays. HPLC analyses showed that crude extracts of *E. coli* DH5α(p11A07) hydrolyzed biuret, while *E. coli* DH5α (pUC18) did not degrade this substrate (Fig. 4B). Ammonia release assays showed that ORF101 hydrolyzed biuret with a specific activity of approximately 3 nmol of ammonia per min per mg of protein (data not presented). The product(s) of biuret hydrolysis by ORF101 was examined by using TLC and HPLC analyses. Allophanate was detected by both methods, with extracts from *E. coli* DH5α (pUC18) showing only limited loss of allophanate, while extracts from *E. coli* DH5α(p11A07), most likely due to the long incubation, no such zone was detected with *E. coli*(pUC18). Results from three replicates. Error bars indicate standard deviations of the means.

FIG. 5. Sequence alignment of ORF101 and ORF102 with members of the amidase protein family. Identical amino acids are boxed. The arrows indicate residues that have been shown to be important for amidase activity in a fatty acid amide hydrolase (FAAH). Residues common to the amidases and aspartic proteases are denoted by the asterisks. The amidase sequences used for the alignment were as follows: ORF102 (nucleotides 104283 to 106100), located downstream of *atzE* on p14D12, was found to have 44% sequence identity to the putative amidase from *Caenorhabditis elegans* (accession number gi:77820); AMD, acetamidase, *Pseudomonas syringae* accession number gi:1079452; FAAH1, fatty acid amide hydrolase, *Rattus norvegicus* (accession number gi:6425411); IAAH, indoleacetamide hydrolase, *Pseudomonas syringae* accession number gi:77820; AMD, acetamidase, *Emericella nidulans* (accession number gi:101782).

FIG. 6. Ammonia released after incubation of biuret with cell extracts from *E. coli* DH5α (p11A07) and *E. coli* DH5α (p14D12) incubated with biuret released three times more ammonia when combined with *E. coli* (p14D12) (producing AtzF) than in reactions containing only biuret hydrolysis. Since AtzF does not hydrolyze biuret or urea, these results confirm that allophanate is the product of biuret hydrolysis by AtzE. Taken together, these results indicate that all genes necessary for the catabolism of atrazine to ammonia and carbon dioxide are localized on pADP-1 and that the last
three genes in the atrazine degradation pathway, \textit{atzD}, \textit{atzE}, and \textit{atzF}, are located in a contiguous cluster in the pADP-1 plasmid. ORF99 (nucleotides 99885 to 100853) had 39% identity to members of the LysR family of transcriptional regulators (50). ORF99 is divergently transcribed from \textit{atzD} and located 166 bp from the start codon of this gene. Moreover, ORF99, \textit{atzD}, \textit{atzE}, and \textit{atzF} have similar mol\% G + C contents (59 to 61%) and codon usage, suggesting that these four genes constitute an operon-like structure on pADP-1. To determine whether the \textit{atzD}, \textit{atzE}, and \textit{atzF} genes are transcribed as a single mRNA, we used RT-PCR and primers designed to amplify contiguous cDNAs between \textit{atzD} and \textit{atzE} and from \textit{atzE} to \textit{atzF}. RT-PCR analyses showed that a single transcript of 2.3 kb was obtained using the \textit{atzD} and \textit{atzE} primers and a 1.1-kb transcript was obtained using the \textit{atzE} and \textit{atzF} primer pair (data not shown). A single mRNA from \textit{atzD} to \textit{atzF} would be approximately 5.2 kb and difficult to amplify. Consequently, two overlapping primer pairs were used. Since these two cDNAs were derived from a contiguous DNA region containing \textit{atzDEF}, this result indicates that these three genes are cotranscribed as a single mRNA in \textit{Pseudomonas} sp. strain ADP.

Other regulatory proteins. Several other ORFs with homology to transcriptional regulators were also identified in different regions of the pADP-1 sequence. ORF45 (nucleotides 45919 to 46494) had 30% identity to the transcriptional regulators of the Tet/Am family (2, 42). ORF66 (65433 to 65999), which is located approximately 4.2 kb downstream of the \textit{atzC} gene, had 67% identity to a putative transcriptional regulator from \textit{Pseudomonas aeruginosa}. Domain analysis done using Pfam showed that this ORF was related to members of the Tet family of transcriptional regulators (2, 42).

Mercury resistance genes. A DNA region with high homology to genes and an operon involved in mercury resistance was identified in the pADP-1 sequence (ORFs 50 to 57, nucleotides 55067 to 59386). This approximately 4.3-kb DNA region contains eight ORFs, each of which had >99% sequence identity to proteins from the mercury resistance operon identified in \textit{Xanthomonas} sp. strain W17 (32) (Table 1). The functionality of the putative mercury resistance operon in pADP-1 was determined by measuring mercuric reductase activity in crude extracts of \textit{E. coli}(pADP-1) strains and by determining growth in LB medium containing different concentrations of HgCl$_2$. Mercuric reductase activity in \textit{E. coli} AD256(pADP-1) and \textit{E. coli} S17-1(pADP-1) was 301 ± 18 and 387 ± 33 nmol/min/mg of protein, respectively, while the specific activity of this enzyme was 10-fold lower in the control strains (21 ± 4 and 30 ± 2 nmol/min/mg of protein for \textit{E. coli} S17-1 and \textit{E. coli} AD256, respectively). In results, ORF94 (nucleotides 94005 to 94928) and ORF95 (94918 to 96039) are homologous to permeases of type E and permeases of type F, respectively. ORF96 (96026 to 97648) had 58% identity to the ATP-binding component of the same type ABC transporter (GenBank accession no. AE004451). In addition, two ORFs (ORF46 and ORF69) had homology to a hypothetical protein and a secondary magnesium/citrate transporter, respectively. These ORFs were classified as transporters based on results obtained from domain searches done using Pfam. ORF46 (46883 to 48223) showed a significant match to a xanthine/uracil permease family of proteins, having many residues conserved in the signature sequence of this family of proteins (1, 19). In addition, hydrophobicity plots of ORF46 predicted 12 transmembrane domains, a characteristic that is commonly observed in the xanthine/uracil permease family of proteins (1, 19). Similarly, hydrophobicity plots of ORF69 (67797 to 68942) showed 12 membrane-spanning domains, and BLAST and Pfam searches showed predicted homology to a variety of transporters.

Hypothetical proteins. Approximately 8% of the predicted ORFs on pADP-1 sequence had homology to conserved hypothetical proteins identified in other genomes, especially to hypothetical proteins from \textit{Pseudomonas aeruginosa}. In an attempt to identify the potential function of these predicted ORFs, sequences were analyzed to identify motifs and conserved sequence domains. ORF58 (nucleotides 59783 to 60556), ORF59 (60661 to 61119), ORF61 (62296 to 62769), ORF63 (63790 to 64593), ORF97 (97655 to 98737), and ORF98 (98776 to 99714) had no significant matches to proteins present in the Prosite, Pfam, PRINTS, and BLOCKS databases. Therefore, the function of these proteins remains unknown. However, ORF62 (62824 to 63771) and ORF30 (25315 to 26307) had a low percentage of identity to many membrane-bound proteins and transporters. The hydrophobicity plots of these ORFs predicted 6 to 10 transmembrane domains and suggested that these ORFs may be membrane-bound proteins or transport proteins. About 2% of the ORFs had no significant homology to any known proteins in the databases.

DISCUSSION

In this study we describe the complete nucleotide sequence of catabolic plasmid pADP-1 from \textit{Pseudomonas} sp. strain ADP. One of the most remarkable features of plasmid pADP-1 is its high degree of relatedness to the 53.3-kb broad-host-range IncP\textsuperscript{B} plasmid pR751, initially isolated from \textit{Enterobacter aerogenes} (59). Nearly one-half of pADP-1 consists of the pR751 backbone (Fig. 2). Members of the IncP plasmid group have been shown to be highly conserved despite the fact that they have been isolated from diverse genera of bacteria in different parts of the world (11, 54, 60). Antibiotic resistance, mercury resistance, and other catabolic genes have previously been identified on diverse IncP plasmids (11, 54). Burlage et al. (11) reported that a 30-kb DNA fragment from pR751 was conserved in the catabolic plasmids pJ4, pSS50, and pSS60. Moreover, antibiotic resistance plasmids pR906 and pR772...
have several loci identical to those found in pR751 (54). Similarly, Tralau and coworkers (60) mapped pTSA from *Comamonas testosteroni* T2, encoding *p*-toluenesulfonate degradation, and reported that the 72.4-kb catabolic plasmid also had a similar backbone structure.

The region of pADP-1 between the *tra* and *trb* operons (nucleotide positions 24062 to 76280) contains the *atzA, atzB,* and *atzC* genes. While our previous data suggested that the gene for cyanuric acid degradation was not located on pADP-1 (17), in the present study we show, by sequence and functional analysis, that *atzD* (ORF100) hydrolyzes cyanuric acid to biuret (Fig. 4A and 7). *AtzD* has 58% sequence identity to *TrzD* from *Pseudomonas* sp. strain NRRLB-12227. *TrzD* has been shown to be part of a composite transposon flanked by two IS1071 elements (60).

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