Molecular Analysis of Isophthalate and Terephthalate Degradation by *Comamonas testosteroni* YZW-D

Yong Zhao Wang, Yiming Zhou, and Gerben J. Zylstra

Center for Agricultural Molecular Biology, Cook College, Rutgers University, New Brunswick, New Jersey

*Comamonas testosteroni* YZW-D was isolated from Passaic River sediment for its ability to degrade isophthalate and terephthalate. Degradation of the two isomeric compounds proceeds via separately inducible catabolic pathways that converge at protocatechuate. Analysis of the catabolic pathways by which these two isomers are degraded demonstrated that a cis-dihydriodiol intermediate is involved in both pathways. The genes for the conversion of isophthalate and terephthalate to protocatechuate were cloned on a single fragment of genomic DNA from *C. testosteroni* YZW-D. The two operons were located by subcloning and mutant complementation experiments. The regions coding for the two degradative pathways were sequenced. Analysis of the nucleotide sequence for the isophthalate degradation operon located genes for a dioxygenase, a transport protein, a cis-dihydriodiol dehydrogenase, and a reductase. Analysis of the nucleotide sequence for the terephthalate degradation operon located genes for a regulatory protein, a transport protein, a dioxygenase large subunit, a dioxygenase small subunit, a cis-dihydriodiol dehydrogenase, and a reductase.

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**Introduction**

Phthalate and phthalate esters are widely used by industry for a variety of applications. The largest use of phthalates is in the manufacture of polymers. For instance, certain synthetic textile fibers, films, plastics, and baked-on paints are composed of polymers formed from phthalates and other materials. In addition, phthalates are used in pesticide carriers, munitions, cosmetics, and insect repellents. Due to their wide use, phthalates are a common contaminant in the environment. Phthalates and phthalate esters have been shown to be teratogenic, having neuromuscular effects and producing congenital malformations. Phthalates also have physiological effects, with certain members of this class acting as central nervous system depressors and others acting as central nervous system stimulants (1). Knowledge of the biochemical, molecular, and physiological mechanisms by which microorganisms degrade the individual members of this class of compounds will aid in the design and implementation of efficient remediation strategies.

**Isolation and Characterization of Strains**

To analyze the degradation of phthalates in more detail, a number of strains that have the capability to degrade one or more phthalate isomers (phthalate, isophthalate, or terephthalate) were isolated. Sediment from the Passaic River in New Jersey was used as the inoculum for enrichment cultures using each of the three phthalate isomers as a carbon source. After a number of serial subcultures, the cells were plated onto solid medium, and isolated colonies were picked and purified. Eight distinct strains that have the capability to grow on one or more of the three phthalate isomers (Table 1) were isolated. For instance, strains A, C, G, and H could grow only on one of the three phthalate isomers, strains D and F could grow on two of the three phthalate isomers, and strains B and E could grow on all three of the phthalate isomers. It is interesting to note that all of these strains were isolated from the exact same location, but they have differing abilities to grow on the aromatic acids phthalate, isophthalate, terephthalate, p-hydroxybenzoate, and benzoate.

Initially, strain D was chosen for an in-depth investigation of isophthalate and terephthalate degradation. The strain is Gram-negative and was classified by the Biolog identification system (Biolog, Inc., Hayward, CA) as *Comamonas testosteroni*. The first experiments were designed to determine if *C. testosteroni* YZW-D possesses a single catabolic pathway for the degradation of both isophthalate and terephthalate or if the strain possesses two separate catabolic pathways, each specific to one of the two isomers. The former hypothesis is true for the TOL plasmid that degrades the two isomeric compounds

| Table 1. Ability of the isolated strains to grow on related aromatic carbon sources. |
|-----------------------------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Carbon source                              | A      | B      | C      | D      | E      | F      | G      | H      |
| Phthalate                                    | -      | +      | -      | -      | +      | +      | +      | -      |
| Isophthalate                                  | -      | +      | +      | -      | +      | -      | -      | +      |
| Terephthalate                                 | +      | +      | -      | +      | -      | +      | -      | -      |
| p-Hydroxybenzoate                             | +      | -      | +      | -      | +      | +      | -      | -      |
| Benzoate                                      | +      | -      | -      | -      | -      | -      | +      | -      |
m-xylene and p-xylene by the same catabolic pathway (2). m-Xylene is degraded through m-toluic acid and 3-methylcatechol, while p-xylene is degraded through p-toluic acid and 4-methylcatechol by TOL-plasmid encoded enzymes. It is possible that a similar catabolic system exists for the degradation of isophthalate and terephthalate (which are structurally analogous to m-toluic acid and p-toluic acid). Oxygen polarographic metabolic assays of C. testosteroni YZW-D, following growth on either isophthalate or terephthalate, showed that two separate catabolic pathways converging at protocatechuate are responsible for isophthalate and terephthalate degradation. Washed cells, following growth on terephthalate, showed oxygen uptake when exposed to terephthalate, but showed no oxygen uptake when exposed to isophthalate. Conversely, washed cells, following growth on isophthalate, showed oxygen uptake when exposed to isophthalate and no oxygen uptake when exposed to terephthalate. However, both sets of cells showed oxygen uptake when exposed to protocatechuate, suggesting that this compound is an intermediate in the catabolic pathway for both isophthalate and terephthalate. Enzyme assays for protocatechuate dioxygenase activity (3) in crude cell extracts, following sonication of the cells, demonstrated that protocatechuate was cleaved at the 4,5 position, one of the defining metabolic characteristics of C. testosteroni.

A number of mutant strains that are blocked in either isophthalate or terephthalate degradation by C. testosteroni YZW-D were constructed. These mutants were constructed by N-methyl-N'-nitro-N-nitroso-guanidine mutagenesis and screened by using the indicator dye 2,3,5-triphenyl-2H-tetrazolium chloride in medium containing succinate plus either isophthalate or terephthalate following established procedures (4). The mutants obtained were screened for enzymatic activity against isophthalate, terephthalate, and protocatechuate. In addition, accumulating oxygenated metabolites were detected using 4-aminantipyridine (5) and high-pressure liquid chromatography (HPLC). Mutants that were missing isophthalate oxygenase activity, terephthalate oxygenase activity, and protocatechuate 4,5-dioxygenase activity were identified. Mutants without the ability to transport terephthalate into the cell were identified by transport assays. These mutants were used in the cloning experiments described in the next section.

A number of mutants that had oxygenase enzyme activity against isophthalate or terephthalate and that accumulated a polar intermediate in the culture medium (as determined by HPLC) were obtained. These mutants were used to accumulate this intermediate for identification to determine the individual steps in the catabolic pathway. Following growth on succinate in the presence of isophthalate, mutant D6 accumulated a single polar metabolite, as detected by HPLC. This metabolite reacted with 4-aminantipyridine only after acidification and heating of the culture supernatant. Since 4-aminantipyridine reacts with phenolic groups, these data suggest the presence of a cis-dihydrodiol compound that does not react with this reagent unless it is dehydrated to a phenolic compound by acidification and heating. Isolation of the suspected cis-dihydrodiol compound from the culture medium was performed by ion exchange chromatography followed by extraction of the appropriate fractions with tetrahydrofuran and evaporation to dryness. Analysis of the extract by HPLC gave a single peak that was lost upon acidification and heating of the extract before the HPLC analysis. Two new peaks appeared in these samples, one of which was identified as m-hydroxybenzoate using a known standard. Derivatization of both compounds with diazomethane, followed by analysis by gas chromatography and mass spectroscopy, indicated that the presence of a compound with the expected molecular mass of a cis-isophthalate dihydrodiol. Analogous experiments with mutant D37 were performed following growth on succinate in the presence of terephthalate. The results were consistent with the accumulation of cis-terephthalate dihydrodiol.

The enzymological and mutant studies of C. testosteroni YZW-D are consistent with the catabolic pathway shown in Figure 1. The initial steps in the degradation of isophthalate and terephthalate proceed by means of separately inducible enzymes. The two pathways converge at protocatechuate, which is the substrate for meta-ring cleavage at the 4,5 position. Further metabolic reactions lead to the production of tricarboxylic acid cycle intermediates.

**Cloning and Nucleotide Sequence of the Genes for Isophthalate and Terephthalate Degradation**

To study the degradation of isophthalate and terephthalate in more detail at the molecular level, the genes for these two catabolic pathways were cloned. A genomic library of C. testosteroni YZW-D was constructed in the cosmid vector pMMP34 (6) using partially MboI-digested genomic DNA. The cosmid library was transferred to the mutant C. testosteroni YZW-D strains and screened for complementation of the mutations. These experiments yielded one cosmid clone that had the ability to complement mutations in the initial steps of the degradation of both isophthalate and terephthalate. A second cosmid clone was isolated that had the ability to complement the protocatechuate 4,5-dioxygenase mutant strains, indicating that the downstream segments of the catabolic
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Pathway are not linked to the initial segments. Fragments of the original cosmid clone were subcloned into the vector pRK415 (7) and analyzed for their ability to complement mutations in either isophthalate or terephthalate degradation. The results (Figure 2) indicate that the genes for isophthalate degradation are encoded by a 4.9 kilobase pair BamHI to SalI fragment, and that the genes for terephthalate degradation are encoded by a 5.9 kilobase pair EcoRI to XbaI fragment.

The nucleotide sequence of the 4.9 kilobase pair BamHI to SalI region of DNA encoding the genes for isophthalate degradation was determined. A cartoon of this nucleotide sequence is presented in Figure 3. The open reading frames were identified by the mutant complementation data and by amino acid sequence homology with proteins of known function. The genes for the dioxygenase and the reductase, components of a presumed multicomponent enzyme system responsible for catalyzing the first step in the catabolic pathway, are located at the beginning and end of the sequenced region. This is unusual since the genes for other aromatic dioxygenase enzyme systems are sequentially arranged in the operon (8). The nucleotide sequence data also indicate that the enzyme consists of a two-component system with a single subunit oxygenase component. The gene for the dehydrogenase, responsible for catalyzing the conversion of cis-isophthalate dihydrodiol to protocatechuic acid, is the third gene in the operon. The second gene in the operon encodes a protein responsible for transport of isophthalate into the cell. No regulatory genes have yet been located for this operon.

The nucleotide sequence of the 5.9 kilobase pair EcoRI to XbaI region of DNA encoding the genes for terephthalate degradation was determined. A cartoon of this sequence is shown in Figure 4. Functions for each of the open reading frames were assigned based on mutant phenotypes, subclone characteristics, and amino acid sequence homology to other proteins of known function. A gene encoding a regulatory protein was identified at the beginning of the sequenced region, transcribed in the opposite direction from the genes encoding the enzymes of the catabolic pathway. That this protein is actually involved in regulation was shown by the ability of a subclone containing only this region of DNA to complement a mutant strain lacking terephthalate degradative ability. The first structural gene in the operon encodes a protein responsible for transport of terephthalate into the cell. A mutant strain complemented by this subcloned region is deficient in terephthalate transport, as determined by transport assays performed with radioactively labeled terephthalate. In addition, an in-frame phoA fusion with this open reading frame was constructed and shown to express PhoA activity. PhoA activity would be possible only if the phoA fusion segment of the protein were present outside the cytoplasmic membrane. The genes for the dioxygenase and the reductase, responsible for catalyzing the first step in terephthalate degradation, are the second, third, and fifth open reading frames in the operon. The second and third genes presumably encode for the two subunits of a dimeric oxygenase component. The gene for the dehydrogenase, responsible for the conversion of cis-terephthalate dihydrodiol to protocatechuic acid, is the fourth open reading frame. This gene order is similar to that seen for the isophthalate degradative operon in terms of the relative positions of the genes for the dioxygenase, dehydrogenase, and reductase. However, the position of the gene responsible for transport of the substrates into the cell is switched with that of the dioxygenase gene between the two operons. The terephthalate degradative operon encodes a two-subunit oxygenase component of the terephthalate dioxygenase multicomponent enzyme, while the isophthalate degradative operon encodes a single subunit oxygenase component of the isophthalate dioxygenase multicomponent enzyme.

Conclusion and Future Directions

The catabolic pathways for isophthalate and terephthalate degradation in C. testosterone YZW-D proceed through a cis-dihydrodiol intermediate to protocatechuic acid. The catabolic pathways for these two isomers are separately regulated, and the enzymes involved are specific to only one isomer. The two operons responsible for these two catabolic pathways are located adjacent to each other in the genome. Nucleotide sequence analysis of the cloned region has located the individual genes and identified their functions. Future work will involve isolation of the enzymes involved and a more in-depth analysis of regulation and transport. In addition, the studies described here form a basis for an analysis of gene evolution and microbial diversity through comparing the molecular aspects of phthalate degradative pathways present in different microorganisms.

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Figure 2. Restriction map of the 18 kilobase pair cloned region of Comamonas testosterone YZW-D genomic DNA that contains the genes for the initial steps in the degradation pathways of isophthalate and terephthalate. The regions of DNA that complement mutations in the isophthalate or terephthalate degradative pathways are indicated.

Figure 3. Nucleotide sequence cartoon of the 4.9 kilobase region coding for isophthalate degradation by Comamonas testosterone YZW-D. The genes and gene products are indicated.

Figure 4. Nucleotide sequence cartoon of the 5.8 kilobase sequenced region coding for terephthalate degradation by Comamonas testosterone YZW-D. The genes and gene products are indicated.
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