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MINIREVIEW

The TOL (pWW0) Catabolic Plasmid

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INTRODUCTION

At present, the best-understood catabolic plasmid is the TOL plasmid, which encodes the enzymes that degrade toluene. The archetype TOL plasmid, pWW0, was first described in 1974 by Williams and Murray (51). Besides toluene degradation, this plasmid has been shown to mediate the degradation of *m*- and *p*-toluate, *m*- and *p*-xylene (54), and related compounds (10, 31) to acetaldehyde and pyruvate through a bifurcating pathway. Although this review will be limited to a description of pWW0, many other TOL plasmids have been described, most of which have similar biochemical pathways and regions of strong DNA homology to pWW0 (29, 30, 50, 52). These plasmids are usually found in *Pseudomonas* species, although one TOL-like plasmid has been found in *Alcaligenes eutrophus* (20); gene expression appears to be limited to *Pseudomonas* and related species (4).

Because it is the best-characterized example of a TOL plasmid, a detailed review of the physical nature of pWW0, its catabolic enzymes, and genetic structure is provided to develop a fundamental understanding of the molecular biology of catabolic plasmids. In addition, a current model for regulation of gene expression is presented, demonstrating the versatility and complexity that is associated with this plasmid as well as the potential complexity of other plasmid-encoded catabolic systems.

PHYSICAL DESCRIPTION

The TOL plasmid pWW0 was originally described in *Pseudomonas arvilla* mt-2, a strain that was later renamed *Pseudomonas putida* (*arvilla*) mt-2 and that is usually referred to as *P. putida* mt-2. The plasmid is self-transmissible and is a member of incompatibility group P-9. It is very large, about 117 kilobase pairs in size, approximately 40 kilobase pairs of which is needed for the catabolic pathway and the regulatory genes. The spontaneous loss of this region, coupled with the loss of degradative capacity, led Chakrabarty et al. (2) to propose that a transposon may be associated with the TOL plasmid; this loss was later attributed to intraplasmidic recombination between two direct repeat sequences which flank the degradative pathway (36). The transposable element that includes the entire pathway has recently been described (49). Transposition events that have been observed indicate that the size of the transposon is approximately 56 kilobase pairs. The genes involved in transposition have also been identified, including the transposase gene, *tnpA*, and two genes that are necessary for resolution, *tnpS* and *tnpT*. In addition, a specific DNA sequence required in *cis* for resolution, the *res* site, has been identified. The spatial arrangement of these genes is shown in Fig. 1.

Very little is known about the TOL plasmid aside from its catabolic region. The locations of the genes for replication and for conjugal transfer have been mapped only roughly (13), and very little is known about either process. However, Bradley and Williams (1) described the pWW0-encoded pilus and suggested that it is constitutively expressed in its *P. putida* host.

CATABOLIC PATHWAYS

The description of the metabolic pathway found on pWW0 was complicated by the presence of other catabolic genes on the chromosome, in particular the enzymes for the degradation of catechol (Fig. 2). Early studies of catechol metabolism in other species demonstrated that catechol could be oxidized either through intradiol fission by catechol 1,2-oxygenase (termed *ortho* cleavage) or through extradiol fission by catechol 2,3-oxygenase (*meta* cleavage) (6, 7). The chromosomal genes of *P. putida* mt-2 encode the *ortho* pathway, while the TOL plasmid encodes the *meta* cleavage pathway (12, 37).

The presence of these two pathways is important to note because different substrates induce different pathways. For instance, benzoate is metabolized by the *meta* pathway, while catechol, though it is an intermediate of toluene degradation, is catabolized by the *ortho* pathway. The description of these two pathways was essential for the elucidation of the TOL-encoded genes, since it was theoretically possible for only a fraction of the pathway to be plasmid encoded. The work of Nakazawa and Yokota (40) was instrumental in describing the differences between the *ortho* and *meta* pathways. The enzymes and chemical intermediates of toluene degradation are shown in Fig. 3.

The catabolic genes of pWW0 are organized into two operons, conveniently referred to as the upper and lower (or *meta*) pathways (Fig. 1). The upper pathway, *xylCAB*, encodes the degradation of toluene and xylenes to benzoate and toluates (17). It is noteworthy that there is a large sequence between the promoter and *xylC* for which no function has yet been described (32). The lower pathway, *xylDLEGFJKIH*, encodes the degradation of benzoate and toluates to acetaldehyde and pyruvate (16) (Table 1).

Only a few of these enzymes have been examined in any detail. Complementation analysis of transposon mutations of *xylD* (toluate 1,2-oxygenase) revealed four complementation groups (19). The first two apparent cistrons constitute a single gene, *xylX*, which encodes a polypeptide of 57 kilodaltons (kDa). The third cistron, *xylY*, encodes a 20-kDa protein; the fourth cistron, *xylZ*, has an unknown function. These subunit sizes are similar to those of other known dioxygenases (11, 48). The nature of the apparent intracis-

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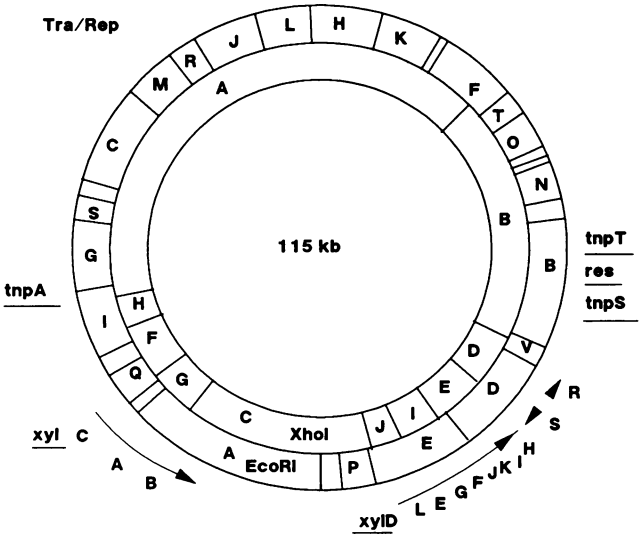

FIG. 1. Map of the TOL plasmid pWW0. Restriction fragments for *Xho*I (inner circle) and *Eco*RI (outer circle) are noted, as are the known genes. For a description of the genes, see the text. Arrows indicate the direction of transcription.

tronic complementation has yet to be determined, but it has been suggested that the *xylX* gene product forms a functional multimer or has two functional domains.

Catechol 2,3-oxygenase (metapyrocatechase), the product of *xylE*, has been purified and characterized (38), and the gene has been sequenced (39). This protein is particularly useful because an assay for XylE enzyme activity is available, and the gene has been incorporated into convenient transcriptional fusion vectors. The enzyme is composed of four identical subunits, each with a molecular mass of 35 kDa, and requires molecular iron for activity. One of the most marked contrasts between pWW0 and other TOL plasmids is that pWW0 has a single metapyrocatechase gene, whereas other plasmids sometimes have two (3). In the latter case, the enzymes might be similar or quite different; the significance of this redundancy is not known.

The *xylF* gene encodes 2-hydroxymuconic semialdehyde hydrolase, which forms one side of the branching catabolic pathway (Fig. 3). This enzyme has been explored in great detail by Duggleby and Williams (9), who were able to purify it to homogeneity. It is functional as a dimer with a total molecular mass of 65 kDa. The branching of the lower pathway was described by Sala-Trepat and Evans (45) by using *Azotobacter* strains. They were able to demonstrate that the pathways rejoined at a common intermediate. An in-depth examination of the branching pathway was reported by Harayama et al. (18). They determined that different starting compounds were metabolized by different branches of the pathway. For instance, *m*-toluate is degraded by the *xylF* pathway, while benzoate and *p*-toluate are degraded by the *xylGHI* branch. Enzyme affinities determine which branch will metabolize the compound; the branching allows an expansion of the substrate range that this organism can utilize. As demonstrated in Fig. 3, the side products of these branches are different, although they both yield the same final product, 2-oxo-4-pentenoate.

GENETIC REGULATION

Two regulatory genes, *xylR* and *xylS*, are now known (53). They are transcribed from physically close but functionally

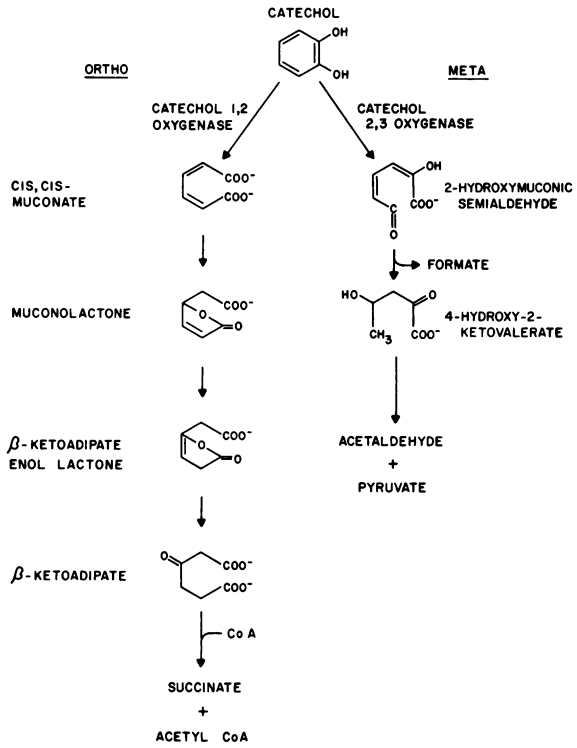

FIG. 2. *ortho* and *meta* cleavage pathways for the degradation of catechol. Chemical intermediate names appear beside the compounds. CoA, Coenzyme A.

divergent promoters (13, 14, 22, 23). Although only a fraction of the *xylR* gene has been sequenced, the size of the XylR protein has been estimated to be 68 kDa (25, 47). The *xylS* gene, encoding a 36.5-kDa protein, has been completely sequenced (26, 35, 47).

The control of the upper and lower pathways of TOL, and of the regulatory genes, is still not fully understood. However, some generalities can now be made about the induction of the pathways, and recent work has allowed speculation about the roles of the regulatory proteins. For example, substrates for the upper-pathway enzymes, such as toluene or *m*-methyl benzyl alcohol, are activators of the pathway when *xylR* is present. In a similar manner, *m*-toluate is both a substrate and an inducer for the lower pathway in conjunction with the *xylS* gene product. However, upper-pathway substrates can activate the lower pathway if both *xylR* and *xylS* are present. A recent report (5) has also demonstrated that the lower pathway can be induced by benzoate alone, without either regulatory protein, which suggests that a chromosomal regulator may be involved.

Attempts to describe induction have been based on genetic studies of the known regulatory genes. An important finding relating to operon control was the demonstration by Dixon (8) that expression of the upper-pathway genes in an *Escherichia coli* host is increased substantially when the host *ntrA* gene is functional. The NtrA protein is a sigma factor that allows certain promoter sequences, notably those involved in nitrogen regulation, to be recognized by RNA polymerase. These promoters typically have very little homology to consensus *E. coli* promoters. Instead, they have discrete areas of homology at the −12 and −24 positions. This finding suggests that control of similar promoters of TOL may be mediated through *ntrA* gene product activity.

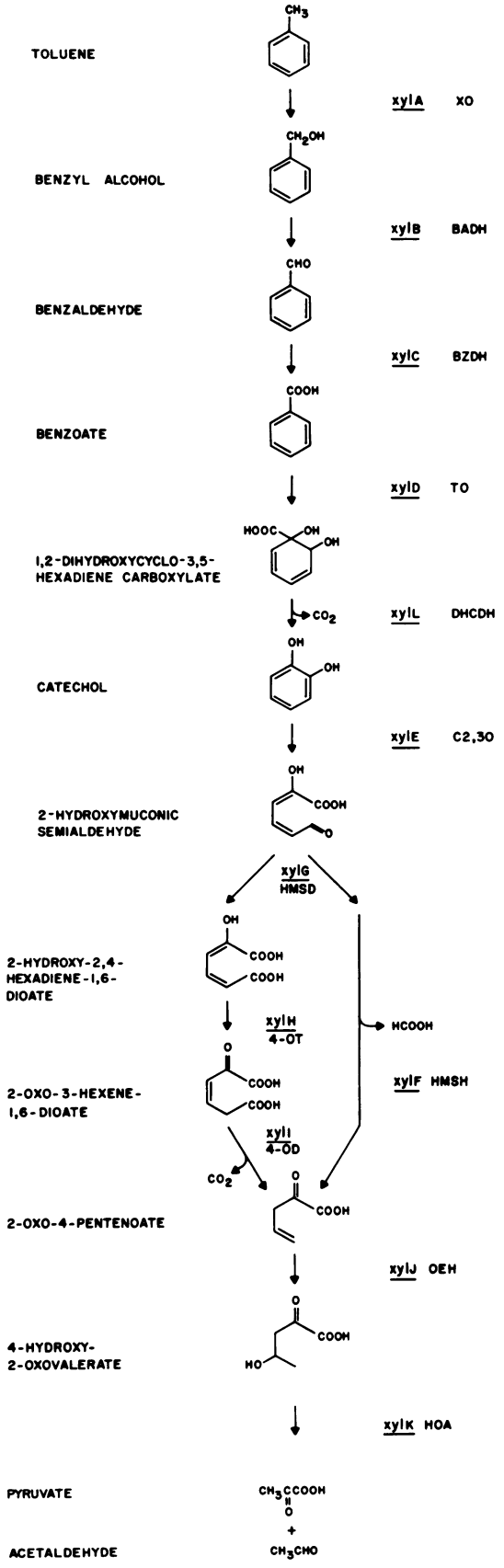

FIG. 3. Pathway for degradation of toluene encoded by *Pseudomonas* plasmid pWW0. Chemical intermediates are listed to the left of the pathway, while the specific degradation genes and the abbreviations of the enzymes that they encode are to the right. For complete names of the enzymes, see Table 1.

TABLE 1. Genes of the TOL degradative pathway

Gene	Enzyme or function	Abbreviation
xylA	Xylene oxygenase	XO
xylB	Benzyl alcohol dehydrogenase	BADH
xylC	Benzaldehyde dehydrogenase	BZDH
xylD	Toluate oxygenase	TO
xylX	Terminal oxidase?	
xylY	Terminal oxidase?	
xylZ	?	
xylE	Catechol 2,3-oxygenase	C2,3O
xylF	2-Hydroxymuconic semialdehyde hydrolase	HMSH
xylG	2-Hydroxymuconic semialdehyde dehydrogenase	HMSD
xylH	4-Oxalocrotonate isomerase	4-OT
xylI	4-Oxalocrotonate decarboxylase	4-OD
xylJ	2-Oxopent-4-enoate hydratase	OEH
xylK	2-Oxyohydroxypent-4-enodate aldolase	HOA
xylL	Dihydroxycyclohexadiene carboxylate dehydrogenase	DHCDH
xylR	Regulation	
xylS	Regulation	

As shown in Fig. 4, these putative promoters are found in the *xylS* (26, 27) and upper-pathway (21) operons and, to a lesser extent, in the lower pathway (24). Description of lower-pathway expression is made more difficult by the presence of two overlapping promoters (34). The lower pathway may require a different sigma factor for expression. The *xylR* promoter is not similar to the other three promoters, although an *E. coli* promoter sequence is present (25, 47); evidence indicates a high constitutive level of expression for *xylR* in *E. coli* and *P. putida* (25). Sequence analysis of several *xyl* genes has found that they have ribosome-binding sites that exhibit good complementarity with both *E. coli* and *Pseudomonas* 16S rRNA, and so control of expression does not appear to be at the level of translation (15). In addition, Dixon's report (8) demonstrated that the *xylR* gene can be replaced with either *ntrC* or *nifA* from *Klebsiella pneumoniae*, genes that promote transcription of nitrogen-regulated operons. This suggests that XylR functions as an activator of transcription.

The available information on regulation was collected by Ramos et al. into a sophisticated model for control of the *xyl* genes (42). In this model, the substrate, e.g. toluene, enters the cell and combines with the XylR protein, which is produced constitutively. This combining changes the protein to an active form that can then bind to the *xylCAB* operon and promote binding of the RNA polymerase and the *ntrA* sigma factor. The products of upper-pathway metabolism are sufficient to induce the activation of the lower pathway by the XylS protein, but it is now apparent that active XylR accomplishes activation of the upper and lower pathways at roughly the same time. XylR achieves this simultaneous activation by promoting transcription at the *xylS* operon,

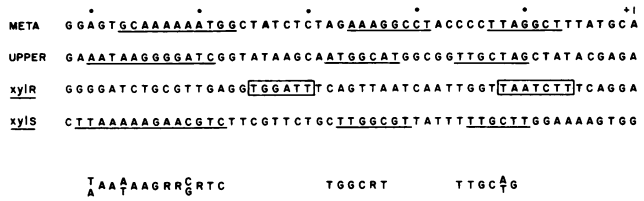

FIG. 4. Promoter regions of the TOL operons. The transcription start site is indicated by +1; 10-base-pair intervals are denoted by dots. The −12, −24, and −45 regions corresponding to those found by Ramos et al. (42) are underlined; consensus sequences appear at the bottom. The letter R denotes a purine. The sequence of *xylR* is from the study by Inouye et al. (25); the *E. coli* promoter sequences are boxed.

again with the *ntrA* sigma factor, which raises the concentration of the *xylS* regulator protein. A proposed operator site for XylR binding is centered at −45 in both the *xylS* and upper-pathway promoters (Fig. 4). The observation that overproduction of XylS is capable of producing constitutive expression of the lower pathway has been demonstrated by no less than three independent groups (27, 35, 46). In each of these studies, XylS overproduction was accomplished with an inducible heterologous promoter, although it now seems that XylR has the same effect, producing a regulatory cascade that allows lower-pathway transcription in the absence of the usual inducer substrates. In keeping with the sequence information for the lower-pathway operon, this stimulation does not require the *ntrA* gene product. It is not known how XylS produces its stimulatory effect, but it has been noted (35) that the amino acid sequence of this regulator bears great similarity to certain sigma factors and contains other sequences that could be DNA-binding domains (26, 35). In support of this latter suggestion is a proposed operator site for XylS binding in the lower-operon promoter region (46).

In the event that a lower-pathway-inducing substance is taken up by the cell, it is hypothesized that it binds directly to the XylS protein, which is made in small constitutive amounts. This converts XylS to an active form, which can then stimulate lower-pathway expression. In order to reconcile these two methods of XylS usage, it is proposed that XylS protein can exist in a dynamic equilibrium in at least two forms. Binding of an inducer substrate can convert the protein to its active form; overproduction of the protein can increase the likelihood that an active molecule will contact the promoter sequence (42).

As expected, a report from a separate group at about this time (28) proposed a model that is essentially identical to this one, which highlights the consensus of thought on this problem. This work is also notable for reporting that *xylR* is an autorepressing gene, even in the presence of an inducing substrate. This information is presented schematically in Fig. 5.

Taken altogether, the Ramos et al. (42) model describes a tightly regulated system with control at the level of transcription. It is interesting that, in the presence of an upper-pathway substrate, the upper and lower pathways are both activated at the same time, in concert with the production of active XylR. It has been hypothesized that this simultaneous activation is advantageous to the cell because it destroys toxic intermediates or because full activation of the pathway would eliminate pools of metabolites that might escape the cell and become a chemoattractant for competing organisms (42).

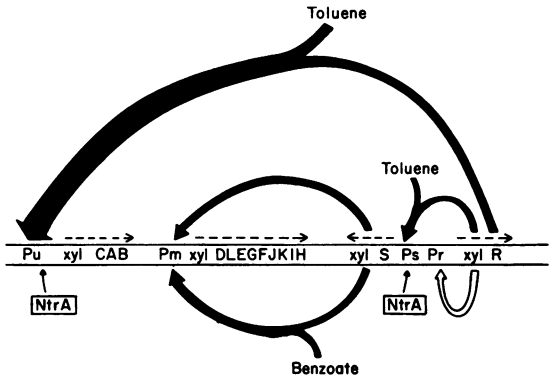

FIG. 5. Proposed model for gene regulation in pWW0. Broken arrows indicate the direction of transcription. Solid arrows represent activation of the indicated operons; open arrows represent repression. Promoters requiring NtrA protein are identified by the labeled box. In this figure, toluene is combining with XylR protein to affect two promoters, P_u and P_s. Increased production of XylS can then activate P_m. Alternatively, benzoate in the presence of the constitutive amount of XylS is able to activate P_m. The *xylR* gene is shown to be an autorepressor. (Adapted from Ramos et al. [42] and Inouye et al. [28].)

DISCUSSION

The many recent advances in the study of the genetic regulation of catabolic pathways, including the many TOL plasmids, has made it possible to use recombinant DNA techniques to produce organisms with unique traits. The degradative pathway of pWW0, both structural and regulatory, is now understood well enough that it can be used to augment other degradation schemes. This was done by Reineke et al. (44) when they transferred pWW0 to *Pseudomonas* strain B13, which contains chromosomally encoded enzymes for the degradation of 3-chlorobenzoate. The transconjugant was able to utilize both toluene and 3-chlorobenzoate as substrates, while spontaneous mutants that activated *xylD* were found that utilized 4-chlorobenzoate. Furthermore, the substrate range was increased in this strain when both *xylD* and *xylL* were activated (33). The regulatory gene *xylS* has also been used to increase the substrate range, taking advantage of the fact that the substrate must combine with the effector molecule to allow activity (41, 43). In this way, rare mutations can be isolated that recognize different substrates.

It is anticipated that further advances in the degradation of recalcitrant compounds will be possible when the other enzymes of TOL, as well as the true nature of the regulatory mechanism, are better understood. This will require more extensive sequencing of the known genes and a study of the structure/function aspects of the enzymes that they encode. In addition, a study of the transport of substrates is required to determine the role, if any, of plasmid-encoded genes in the uptake process.

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