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Transposon Mutagenesis Analysis of *meta*-Cleavage Pathway Operon Genes of the TOL Plasmid of *Pseudomonas putida* mt-2

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Hybrid plasmids containing the regulated *meta*-cleavage pathway operon of TOL plasmid pWWO were mutagenized with transposon Tn*1000* or Tn*5*. The resulting insertion mutant plasmids were examined for their ability to express eight of the catabolic enzymes in *Escherichia coli*. The physical locations of the insertions in each of 28 Tn*1000* and 5 Tn*5* derivative plasmids were determined by restriction endonuclease cleavage analysis. This information permitted the construction of a precise physical and genetic map of the *meta*-cleavage pathway operon. The gene order *xylD* (toluate dioxygenase), *L* (dihydroxycyclohexidiene carboxylate dehydrogenase), *E* (catechol 2,3-dioxygenase), *G* (hydroxymuconic semialdehyde dehydrogenase), *F* (hydroxymuconic semialdehyde hydrolase), *J* (2-oxopent-4-enoate hydratase), *I* (4-oxalocrotonate decarboxylase), and *H* (4-oxalocrotonate tautomerase) was established, and gene sizes were estimated. Tn*1000* insertions within catabolic genes exerted polar effects on distal structural genes of the operon, but not on an adjacent regulatory gene *xylS*.

The TOL plasmid pWWO (115 kilobases [kb]) of *Pseudomonas putida* mt-2 (10, 19, 20) encodes a set of inducible enzymes required for the complete degradation of toluene, *m*- and *p*-xylene, 3-ethyl toluene, and 1,2,4-trimethyl benzene (10, 22). These compounds are oxidized to the corresponding carboxylic acids, i.e., benzoate, *m*- and *p*-toluate, 3-ethyl benzoate, and 3,4-dimethylbenzoate, respectively, which are subsequently degraded to short-chain carboxylic acids, pyruvate, and aldehydes via a *meta*-cleavage pathway (Fig. 1).

The genes of the *meta*-cleavage pathway enzymes are organized into a single regulatory unit that is controlled by the products of two regulatory genes, *xylS* and *xylR* (21). Previous Tn*5* transposon mutagenesis and gene cloning studies (3, 4, 8) demonstrated that genes of the initial enzymes of the *meta*-cleavage pathway (*xylDEGF*) and regulatory genes *xylS* and *xylR* are present on two contiguous *Sst*I fragments of the plasmid (*Sst*I-C and *Sst*I-D).

The genetics of the TOL plasmid catabolic pathway are still poorly characterized. With the increasing interest in this pathway for basic studies of gene expression in *Pseudomonas* spp. (N. Mermod, P. Lehrbach, W. Reineke, W. Knackmuss, and K. Timmis, submitted for publication), the construction of hybrid pathways (14), and the generation of bacteria able to carry out specific chemical transformations (13), the need for a detailed genetic analysis has become urgent.

We report here the use of transposon mutagenesis to order and locate precisely most of the *meta*-cleavage pathway structural genes. This study was carried out with *Escherichia coli*, which expresses TOL genes poorly from the native promoters because these promoters function only weakly in *E. coli* (8; Mermod et al., submitted for publication), but with which genetical analysis can be readily carried out.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains were *E. coli* K-12 derivatives ED2196 (F^- *his trp tsx spc nalA* Δ*lacX74*) (5), S605 (F^- *thr leu thi lacY tonA supE met*::Tn*5*) (17), and RB308 (F^+ *thi leu deoC lacY thyA*). (G. Boulnois, this laboratory). Plasmids pMT057 (12) and pPL392 (this study; Fig. 2) are hybrids of pBR322 (1), whereas pKT502 is a pKT230 hybrid containing the *Xho*I-I fragment of pWWO-161 (3). The Tn*5* and Tn*1000* insertion derivatives of pMT057 and pPL392 are described below.

Media. The media used in this study have been described previously (11, 14). Selection of antibiotic-resistant clones was done by plating bacterial suspensions of Luria agar containing antibiotics at the following concentrations (in micrograms per milliliter): ampicillin, 100; kanamycin, 25; streptomycin, 20; and nalidixic acid, 100.

Preparation of cell extracts and enzyme assays. Cells for enzyme assays were grown in Luria broth at 30°C to late exponential growth phase. Where appropriate, benzoate (5 mM) or *m*-toluate (5 mM) was added to the medium as an inducer. Cell extracts for assays of catechol 2,3-dioxygenase (C23O), 2-hydroxymuconic semialdehyde hydrolase (HMSH), and 2-hydroxymuconic semialdehyde dehydrogenase (HMSD) were prepared and assayed as described previously (16).

Crude extracts for the assay of 4-oxalocrotonate tautomerase (4OT), 4-oxalocrotonate decarboxylase (4OD), and 2-oxopent-4-enoate hydratase (OEH) were prepared by sonicating cells suspended in 100 mM Tris-hydrochloride (pH 7.4) containing 2 mM 2-mercaptoethanol. Assays of 4OT and OEH were carried out as described previously (2, 16), except that 10 mM potassium phosphate buffer (pH 7.3) and 10 mM potassium phosphate buffer (pH 7.3) containing 0.33 mM $MnCl_2$ were used as reaction buffers, respectively. Assay of 4OD was carried out in 100 mM Tris-hydrochloride (pH 7.4) containing 3.3 mM $MgSO_4$ and 67 μM 4-oxalocrotonate. Unreacted 4-oxalocrotonate was assayed by measuring the absorbance at 350 nm after mixing 1.4 ml of the enzyme reaction mixture with 1.6 ml of 0.3 M NaOH–16 mM EDTA. Toluate 1,2-dioxygenase (TO) activity was assayed indirectly as the stimulation of oxygen uptake by whole cells after the addition of *m*-toluate (5 mM); measurements were made at room temperature by using an oxygen electrode. For cells which had low TO activity, 33 mM NaCN was included in the buffer to inhibit endogenous respiration.

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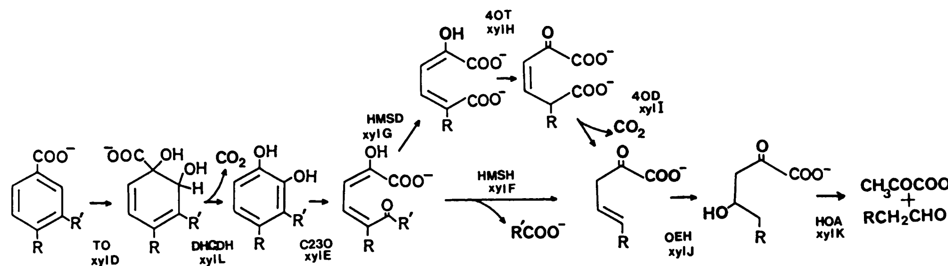

FIG. 1. *meta*-Cleavage pathway specified by TOL plasmid pWWO of *P. putida* mt-2. HOA, 2-Oxo-4-hydroxypentonate aldolase; other enzyme abbreviations are defined in the text. *xylD* to *xylL* are designations of the structural genes of the catabolic enzymes. Initial compounds: R=R′=H, benzoate; R=H,R′=CH_3, *m*-toluate; R=CH_3,R=H, *p*-toluate.

NaCN at this concentration did not affect the activity of TO (Harayama, unpublished data).

Protein concentrations were measured with a Bio-Rad protein assay kit, and all enzyme activities are expressed as nanomoles of substrate consumed per minute (= milliunits) per milligram of protein.

Chemicals. 2-Hydroxymuconic semialdehyde was prepared from catechol by using a cell extract of *P. putida* KT2442(pKT502) (3). The enzymatic synthesis of 2-oxopent-4-enoate from DL-allylglycine was done as previously described (2). The potassium salt of diethyl 2,4-hexadiene-5-hydroxy-1,6-dioate was synthesized as previously described (18), and 4-oxalocrotonic acid was obtained from this compound by the method of Lapworth (11).

Genetic methods. Insertion (Tn*1000*) mutagenesis of plasmid pPL392 was carried out by F-mediated mobilization of pPL392 as previously described (6). Tn*5* insertion derivatives of pMTO57 were isolated by using strain S605 as previously described (17).

DNA manipulations. Methods for plasmid isolation, transformation, cleavage by restriction endonucleases, agarose gel electrophoresis, and gene cloning were as previously described (4, 12, 14). Rapid preparation of plasmid DNA was by the method of Holmes and Quigley (7).

Analytical method. Preparation of samples for thin-layer chromatography and identification of mutants which accumulate dihydroxycyclohexidioene carboxylate were as previously described (14).

RESULTS

Transposon mutagenesis of hybrid plasmids carrying the regulated *meta*-cleavage pathway region of TOL. Plasmid pMTO57 is a pBR322 hybrid containing a 23.5-kb *Hin*dIII fragment of TOL plasmid pWWO (12). Plasmid pPL392 is a derivative of pMTO57 deleted of one *Eco*RI fragment and containing a 16.4-kb fragment of TOL (Fig. 2). From previous studies (3, 4), it is known that the TOL DNA segments in these plasmids carry the whole *meta*-cleavage pathway operon as well as its regulatory gene *xylS*. Plasmid pPL392 was chosen for the generation of a collection of Tn*1000* insertion derivatives, whereas pMTO57 was used to obtain a series of Tn*5* mutant derivatives.

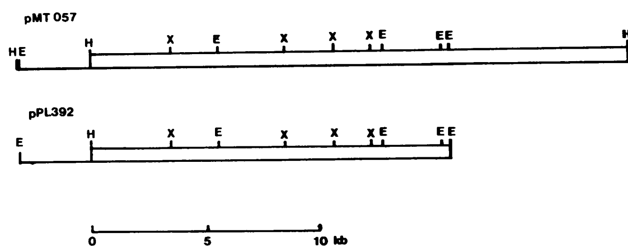

FIG. 2. Physical maps of plasmids pMTO57 and pPL392. Restriction endonuclease cleavage sites for *Eco*RI (E), *Hin*dIII (H), and *Xho*I (X) are indicated. The open boxes represent pWWO DNA, and thin lines represent pBR322.

To determine the physical location of each insertion in pMTO57::Tn*5* and pPL392::Tn*1000* mutant plasmids, plasmid DNA from each derivative was isolated and analyzed by cleavage with restriction endonucleases *Eco*RI, *Hin*dIII, and *Xho*I. The sites of the Tn*1000* and Tn*5* insertions in the mutant plasmids described in the present study are shown in Fig. 3.

Enzyme induction studies with transposon insertion derivatives: definition of the catabolic gene region. The activities of TO, C23O, HMSH, HMSD, 4OT, 4OD, and OEH were measured in cells containing mutant plasmid derivatives (Table 1). Correlation of the physical locations of the insertions with the loss or alteration in expression of the catabolic genes defined two DNA segments (11 and 1 kb) involved in catabolic gene function.

The 11-kb segment corresponded to that of the structural gene region extending from *xylD2057*::Tn*1000* to

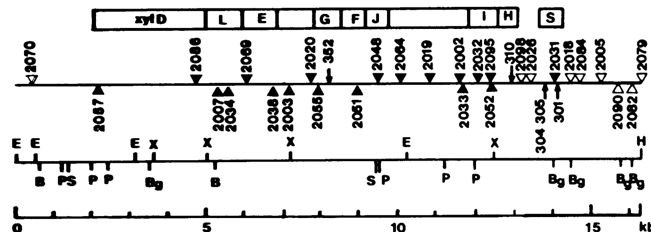

FIG. 3. Physical and genetic map of the TOL *meta*-cleavage pathway genes and locations of transposons Tn*1000* and Tn*5* in mutant plasmids. The location and orientation of 28 independent Tn*1000* (△, ▲) and 5 Tn*5* (↑) insertions within a 16.4-kb segment of pWWO DNA are shown. The sites within Tn*1000* and Tn*5* are taken from Guyer (6) and Jorgensen et al. (9), respectively. Insertions which affect (▲, ↑) or do not affect (△) catabolic functions or their expression are indicated. Restriction endonuclease cleavage sites: *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), *Sst*I (S), and *Xho*I (X).

xylH310::Tn*5*. The gene order in this region was defined as *xylDLEGFJIH* (Fig. 3).

The mutations in two pPL392::Tn*1000* derivatives, *2057* and *2086*, which failed to express any detectable TO activity, were located within a 2.6-kb region of the TOL DNA segment. A preliminary complementation analysis has defined at least four complementation groups within this region (Harayama, unpublished data) and demonstrated that several genes are involved in the expression of TO activity. Two insertions, *xylL2007* and *xylL2034*, caused a lowering of TO activity; unlike the two previous insertions, they caused the accumulation of the metabolite dihydroxycyclohexidione carboxylate in culture supernatant fluids (data not shown). This indicates that the insertions are located within the dihydroxycyclohexidione carboxylate dehydrogenase (DHCDH) gene (*xylL*). The *xylE* (C23O) gene was defined by two insertions, *xylE2069* and *xylE2038*. The location and size (912 base pairs) of *xylE* have been previously determined by DNA sequencing (15, 23).

We placed the *xylG* gene (HMSD) downstream of mutation *xyl-2020* (because although this mutation did not inactivate *xylG*, it severely reduced its expression) and upstream of *xylF2051*::Tn*1000* (because the latter had no effect on HMSD activity). Similarly, we placed *xylF* (HMSH) and *xylJ* (OEH) next to *xylG*. The *xylI* gene (4OD) was placed ca. 2 kb downstream of *xylJ* and *xylH* downstream of *xylI*.

On the basis of our mapping data, we were able to estimate the following maximum sizes (in kilobases) for the catabolic genes: *xylD*, 3.3; *xylL*, 1.1; *xylE*, 0.9; *xylG*, 1.2; *xylF*, 1.5; *xylJ*, 1.1; *xylI*, 1.4; and *xylH*, 0.8.

Location of regulatory gene *xylS*. One Tn*1000* and three Tn*5* insertions, which mapped between coordinates 13.5 and 14.5 kb on the map (Fig. 3), abolished inducibility of C23O by *m*-toluate and benzoate and, hence, defined the regulatory gene *xylS*. Regulatory mutants previously obtained by Tn*5* mutagenesis and whose phenotypes were examined with *P. putida* were defective for induction by *m*-toluate but not by benzoate (4). The difference in phenotypes between the mutants obtained in this study and those previously isolated is currently under investigation.

DISCUSSION

In this study we precisely localized the genes of eight enzymes of the TOL plasmid-specified *meta*-cleavage path-

TABLE 1. Induced activities[a] of TOL plasmid catabolic enzymes in *E. coli* K-12 cells containing plasmid pMT507 or pPL392 and their insertion mutant derivatives

Plasmid or insertion derivative	Enzyme activity[b]								
	TO	C230			HMSD	HMSH	OEH	4OD	4OT
		Uninduced	*m*-toluate	Benzoate					
pPL392 (wild type)	240 (<0.2)	30	780	670	19	14	3,200	36	660
pMT057 (wild type)		29	660	650	11	17			
2070::Tn*1000*		9	390						
xylD2057::Tn1000	<0.2	1	<1						
xylD2086::Tn*1000*	<0.2	3	2						
xylL2007::Tn*1000*	20 (<0.2)	15	24						
xylL2034::Tn*1000*	4.5	100	110	70					
xylE2069::Tn*1000*		<1	<1	<1	2.5	0.7			
xylE2038::Tn*1000*	14.5 (<0.2)	<1	<1	<1					
xyl-2003::Tn*1000*		22	320	630	<0.1	0.3		1.8	9.6
xyl-2020::Tn*1000*		37	210	410	0.3	<0.1		0.4	18
xylG2055::Tn*1000*		34	420	320	<0.1	0.3			
xylG352::Tn*5*		4	42		<0.1	0.4			
xylF2051::Tn*1000*		25	370	500	10	<0.1	<30	1.6	4.8
xylJ2048::Tn*1000*		13	120	64	2.0	2.3	<30		
xyl-2064::Tn*1000*		15	370	57	10	10	860	<0.4	7.2
xyl-2019::Tn*1000*		12	360	83			1,500		
xyl-2002::Tn*1000*		6	310	71				1.6	4.4
xyl-2033::Tn*1000*		13	640	230			1,500	2.2	2.4
xylI2032::Tn*1000*		13	390	45			1,900	<0.4	<1
xylI2095::Tn*1000*		15	430	48			1,700	<0.4	3.0
xylI2052::Tn*1000*		14	230	72			1,100	<0.4	4.6
xylH310::Tn*5*		10	250					69 (1.7)	<1
2098::Tn*1000*		24	260	650			1,400	26	220
2026::Tn*1000*		8	440	490				22	120
xylS305::Tn*5*		7	5	3					
xylS304::Tn*5*		9	10	3					
xylS2031::Tn*1000*		11	15	12					
xylS301::Tn*5*		11	9	6					
2018::Tn*1000*		10	260	460					
2084::Tn*1000*		6	220	370					
2005::Tn*1000*		10	640	570					
2090::Tn*1000*		13	330	460					
2082::Tn*1000*		10	300	360					
2079::Tn*1000*		9	510	480					

[a] *m*-Toluate (5mM) served as the inducer except for C23O, where both uninduced and induced (*m*-toluate or benzoate, both 5 mM) levels of enzyme activity were measured.

[b] Nanomoles of substrate consumed per minute per milligram of protein. Values in parentheses indicate levels in uninduced cells.

way and the *xylS* regulatory gene. Our map had two major gaps to which we cannot presently assign any catabolic gene. Preliminary experiments suggest that the largest gap, 2 kb, between the *xylJ* and *xylI* genes, may contain *xylK*, the structural gene of 4-hydroxy-2-oxovalerate aldolase (Harayama, unpublished data). However, the nature of the determinant(s) located in the gap (0.8 kb) between the *xylE* and *xylG* genes is not presently clear.

The Tn*1000* insertion mutations had a strong polar effect on distal genes. The simplest explanation for this is that the catabolic genes form a single operon which is transcribed as a unit from a site or sites (Mermod et al., submitted for publication) located between insertions *2070*::Tn*1000* and *xylD2057*::Tn*1000* and that transcripts initiated from this site (or these sites) terminate within the Tn*1000* element in mutant derivatives. The possibility that mutations in genes of initial enzymes of the pathway switch off expression of other genes by preventing the formation of metabolites essential for induction of these genes would seem to be excluded by the finding that the operon is fully inducible in a mutant derivative of plasmid pPL392 deleted of the *xylD* gene (Harayama, unpublished).

A number of the mutations obtained caused what seemed to be nonspecific reductions in the activities of several enzymes. For example, bacteria carrying plasmids with mutations in *xylL* or *xylE* showed low induced activities of TO; this might be a consequence of a physiological imbalance caused by their cultivation in the presence of the pathway substrate. For example, the level of NADH, a cofactor of TO, may be low in *xylL* mutant cells because recycling of NADH by DHCDH does not occur. Moreover, accumulation of the toxic product catechol may inhibit functional expression of TO in *xylE* mutants. Furthermore, the *xylJ2048*::Tn*1000* mutation reduced to 20% of their normal levels the activities of the enzymes C23O, HMSD, and HMSH, which are encoded by upstream genes. The reason for this is not clear. Finally, insertions between *xylJ* and *xylH*, but not insertions upstream of *xylJ*, generally reduced the induction of synthesis of C23O by benzoate but not by *m*-toluate. This may suggest that inactivation of the oxalocrotonate branch and the resulting metabolism of benzoate via the hydrolytic branch (not the usual route; Harayama, Mermod, Lehrbach, and Timmis, submitted for publication) is the cause. Metabolism of *m*-toluate via the hydrolytic branch results in the production of acetate, which is readily metabolized by *E. coli*. In contrast, the metabolism of benzoate by this route results in the production of formate, which is toxic to this bacterium and which may prevent functional expression of C23O.

The detailed characterization of gene organization of the *meta*-cleavage pathway operon carried out in this study will enable construction in vitro of specific mutant genes, the subcloning of a specific gene(s), and gene transfer to other bacterial species. Such experimental manipulations will permit further characterization of the catabolic pathway and the construction of strains that accumulate pathway intermediates of interest (and their analogs) or that exhibit increased catabolic activities (14).

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