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Roles of the Divergent Branches of the *meta*-Cleavage Pathway in the Degradation of Benzoate and Substituted Benzoates

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The TOL plasmid-specified *meta*-cleavage pathway for the oxidative catabolism of benzoate and toluates branches at the ring cleavage products of catechols and reconverges later at 2-oxopent-4-enoate or its corresponding substituted derivatives. The hydrolytic branch of the pathway involves the direct formation of 2-oxopent-4-enoate or its derivatives, whereas the oxalocrotonate branch involves three enzymatic steps effected by a dehydrogenase, an isomerase, and a decarboxylase, which produce the same compounds. Evidence is presented which shows that benzoate and *p*-toluate can, under certain circumstances, be catabolized by the hydrolytic branch. However, in a fully functional pathway, only *m*-toluate is dissimilated via this branch, and benzoate and *p*-toluate are catabolized almost exclusively by the oxalocrotonate branch. The biochemical basis of this selectivity was found to reside in the high affinity of the dehydrogenase for ring fission products derived from benzoate and *p*-toluate and its inability to attack the ring fission product derived from *m*-toluate. Although isomerization of 4-oxalocrotonate occurs spontaneously in vitro, enzymatic isomerization was found to be essential for effective functioning of this branch of the pathway in vivo.

Biochemical pathways for the dissimilation of organic compounds constitute a crucial component of the carbon cycle. As a result of their production of relevant catabolic enzymes, soil and water microorganisms are collectively able to catabolize and use as sources of carbon and energy the vast majority of natural and man-made organic compounds. Although these organisms possess a variety of different enzymes for the initial attack of diverse compounds, their catabolic pathways are generally convergent and channel substrates to a limited number of key intermediates, which are then metabolized further by central pathways. For example, a large proportion of different aromatic compounds are converted to one of a few aromatic ring cleavage substrates, such as catechol, gentisate, protocatechuate, and derivatives thereof (31, 34). The benefits of channeling diverse compounds into a few central pathways, namely, a reduced genetic load, the simplification of regulatory circuits, and the economisation of energy, are clearly of major advantage to soil microbes, which often find themselves in unfavorable environments containing low concentrations of carbon sources suitable for growth.

Despite the tendency to converge in catabolic pathways, divergence is nevertheless observed, reflecting the fact that the substituents on some substrates are incompatible with one or more enzymes of particular catabolic routes. For example, the soil bacterium *Pseudomonas putida* containing the TOL plasmid pWWO, which encodes the catabolism of toluene and related aromatic hydrocarbons via the corresponding carboxylic acids and catechols (38, 39, 41), may attack catechols by *ortho* fission, i.e., intradiol cleavage of the aromatic ring, or by *meta* fission, i.e., cleavage between one of the two hydroxyls and an adjacent free carbon (27, 28, 39). Chlorocatechols are generally degraded by an *ortho* fission pathway; if they are subjected to *meta* cleavage, toxic or dead-end intermediates are formed (32). On the other hand, methyl catechols are usually degraded by *meta* cleavage; the *ortho*-cleavage enzyme catechol 1,2-dioxygenase has a very low affinity for methyl catechols or produces dead-end intermediates.

Within the *meta*-cleavage pathways specified by number of plasmids (Fig. 1), including TOL and NAH7 (which specifies the degradation of naphthalene via salicylate and catechol; 43), there exists a further divergence of the pathway at the ring cleavage product and subsequent reconvergence at 2-oxopent-4-enoate or its substituted derivatives (compounds VIII of Fig. 1). The hydrolytic branch of the *meta*-cleavage pathway converts the ring cleavage products (compounds IV) directly to 2-oxopent-4-enoate or its derivatives (compounds VIII; 4, 7, 13, 15, 23, 33) through the action of the enzyme hydroxymuconic semialdehyde hydrolase (HMSH), whereas the 4-oxalocrotonate branch involves the formation of 2-hydroxy-hexa-2,4-diene-1,6-dioate (enol form of 4-oxalacrotonate or its substituted derivatives; compounds V) by the NAD^+-dependent 2-hydroxymuconic semialdehyde dehydrogenase (HMSD), which is then converted to 2-oxopent-4-enoate or its derivatives (compounds VIII) by two enzymatic steps catalyzed by 4-oxalocrotonate isomerase (4OI; formerly called 4-oxalocrotonate tautomerase) and 4-oxalocrotonate decarboxylase (8, 27, 29, 35).

The two branches of the *meta*-cleavage pathway metabolize different catechol derivatives; 2-hydroxy-6-oxohepta-2,4-dienoate (compound IV; R = H, R′ = CH_3), the *meta*-fission product of 3-methylcatechol (compound III; R = H, R′ = CH_3), is not metabolized via the dehydrogenase since this compound does not possess an oxidizable aldehyde. In contrast, 2-hydroxymuconic semialdehyde (compound IV; R = R′ = H) or 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate (compound IV; R = CH_3, R′ = H), the *meta*-cleavage products of catechol (compound III; R = R′ = H), and 4-methylcatechol (compound III; R = CH_3, R′ = H), each can be degraded by both branches, although HMSD activity was found to be several times higher than that of HMSH in TOL plasmid-carrying cells of *P. putida* (27). From these observations, it was proposed that whereas *m*-toluate is exclusively metabolized via the hydrolytic branch, benzoate

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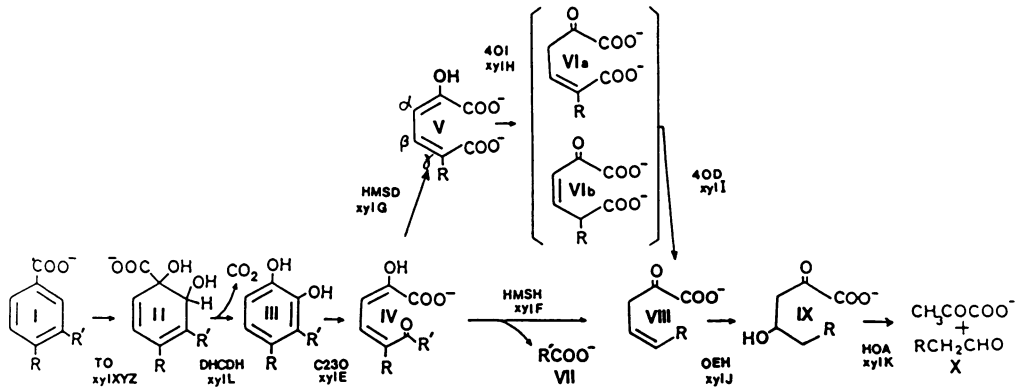

FIG. 1. *meta*-Cleavage pathway for the catabolism of *m*-toluate, *p*-toluate, and benzoate encoded by TOL plasmid pWWO of *P. putida* mt-2. Enzyme abbreviations: TO, toluate 1,2-dioxygenase; DHCDH, 1,2-dihydroxycyclohexa-3,5-diene-carboxylate dehydrogenase; C230, catechol 2,3-dioxygenase; 4OD, 4-oxalocrotonate decarboxylase; OEH, 2-oxopent-4-enoate hydratase; HOA, 4-hydroxy-2-oxovalerate aldolase. *xylE* to *xylZ* are designations of the structural genes for the catabolic enzymes. Compounds: for R = H, R′ = H, (I) benzoate, (II) 1,2-dihydroxycyclohexa-3,5-diene-carboxylate, (III) catechol, (IV) 2-hydroxymuconic semialdehyde, (V) 2-hydroxy-hexa-2,4-diene-1,6-dioate (enol form of 4-oxalocrotonate), (VIa) 2-oxohex-4-ene-1,6-dioate (keto form of 4-oxalocrotonate), (VIb) 2-oxohex-3-ene-1,6-dioate, (VII) formate, (VIII) 2-oxopent-4-enoate, (IX) 4-hydroxy-2-oxovalerate, (X) acetaldehyde; for R = H, R′ = CH_3, (I) *m*-toluate, (II) 1,2,-dihydroxy-3-methylcyclohexa-3,5-diene-carboxylate, (III) 3-methylcatechol, (IV) 2-hydroxy-6-oxohepta-2,4-dienoate, (VII) acetate, (VIII) 2-oxopent-4-enoate, (IX) 4-hydroxy-2-oxovalerate, (X) acetaldehyde; for R = CH_3, R′ = H, (I) *p*-toluate, (II) 1,2,-dihydroxy-4-methylcyclohexa-3,5-diene-carboxylate, (III) 4-methylcatechol, (IV) 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate, (V) 2-hydroxy-5-methyl-hexa-2,4-diene-1,6-dioate, (VIa) 5-methyl-2-oxohex-4-ene-1,6-dioate, (VIb) 5-methyl-2-oxohex-3-ene-1,6-dioate, (VII) formate, (VIII) 2-oxo-*cis*-hex-4-enoate, (IX) 4-hydroxy-2-oxohexanoate, (X) propionaldehyde.

and *p*-toluate could be catabolized by either branch, although they would be preferentially metabolized via the 4-oxalocrotonate branch (27). However, the recent isolation of mutant derivatives of TOL plasmids that permitted *P. putida* host bacteria to metabolize *m*-toluate but not *p*-tolutate (16, 42) seemed to contradict this proposal and suggested that at least *p*-toluate must be catabolized exclusively via the oxalocrotonate branch.

To clarify the roles of the divergent branches of the *meta*-cleavage pathway, we have isolated mutants defective in one or the other branch and have analyzed their phenotypes. Furthermore, since the enol-keto rearrangement occurs spontaneously in vitro, we have examined the requirement for 4OI in the oxalocrotonate pathway. The results reported here define the roles of the two branches of the catechol *meta*-cleavage pathway and demonstrate that the enzyme 4OI is essential for effective functioning of the oxalocrotonate branch.

MATERIALS AND METHODS

Bacterial strains, plasmids, and genetic manipulations. The strains used were *Escherichia coli* LE392 (F⁻ *metB1 lacY1 galK2 galT22 trpR55 supE44 supF58 hsdR514*; 24) and ED2196 (F⁻ *his trp tsx rpsE gyrA* Δ *lacX74*; 19) and *P. putida* PaW94 (41), which is plasmid free and defective in benzoate 1,2-dioxygenase. The pNM72 plasmid, which carries a complete set of *meta*-cleavage pathway genes, has been described previously (26). This plasmid was mutagenized by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, and a derivative was obtained, pGSH2224, that no longer permitted host PaW94 to grow on *m*-toluate, although it could grow on benzoate and *p*-toluate. Other plasmids are described below and in Fig. 2. The method for selection of mutant plasmids that express constitutively genes of *meta*-cleavage enzymes is described elsewhere (26). pKT231-based hybrid plasmids were transferred from *E. coli* LE392(RP1) to *P. putida* PaW94 by RP1-mediated mobilization as described previously (2). Mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine was carried out by the method of Adelberg et al. (1). L broth and minimal medium for the cultivation of *P. putida* have been described (16). Plasmid DNA was isolated by the methods of Hansen and Olsen (18) and Clewell and Helinski (10). Methods for digestion with restriction endonucleases, analysis by electrophoresis through gels of

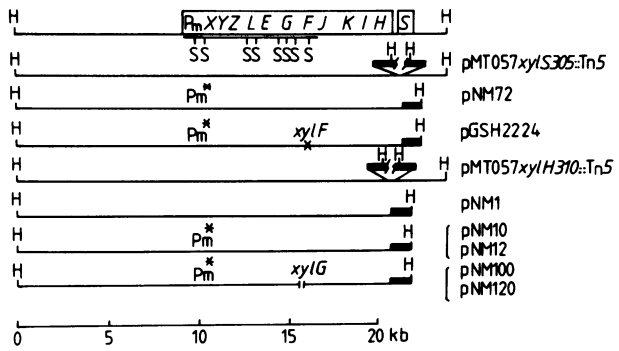

FIG. 2. Physical and genetic map of the TOL plasmid *meta*-cleavage operon region and structure of hybrid plasmids. The *meta*-cleavage operon gene order is taken from previous publications (18, 19). pMT057 *xylS305* and pMT057 *xylH310* are Tn*5* insertion mutants which were obtained in a previous study (18). Symbols: (——) pWWO DNA, (■) Tn*5* DNA. Vector sequences are not shown. The pNM72 plasmid is a pKT231-based hybrid plasmid carrying the large *Hin*dIII fragment of pMT057 *xylS305* and a constitutive promoter (Pm*) which effects expression of the *meta*-cleavage pathway genes (25), whereas the pGSH2224 plasmid is an *xylF* derivative of pNM72. Sizes of *Sal*I (S) fragments are, from left to right, 0.45, 2.3, 0.5 (or 0.52), 1.1, 0.52 (or 0.5), 0.48, and 1.2 kilobases. The 0.48-kilobase *Sal*I fragment containing part of the *xylG* gene is absent from the pNM100 and pNM120 plasmids. H, *Hin*dIII cleavage sites; *xylS*, gene for the positive regulator of the *meta*-cleavage operon; P_m, operon promoter; P_m*, constitutive promoter. See Fig. 1 for nomenclature of other genes.

TABLE 1. Activities of *meta*-cleavage pathway enzymes in *P. putida* PaW94 carrying hybrid plasmids which express constitutively the *meta*-cleavage pathway genes

Strains[a]	Growth phenotype	Enzyme activities[b]				
		HMSH	HMSD	C23O	4OI	4OD
PaW94(pNM10)	*m*-Tol$^+$ *p*-Tol$^-$ Ben$^-$	54	37	1,000	1	NE
PaW94(pNM100)	*m*-Tol$^+$ *p*-Tol$^+$ Ben$^+$	140	7	3,000	1	NE
PaW94(pNM12)	*m*-Tol$^+$ *p*-Tol$^-$ Ben$^-$	110	80	3,000	1	100
PaW94(pNM120)	*m*-Tol$^+$ *p*-Tol$^+$ Ben$^+$	110	2	3,000	1	130
PaW94(pNM72)	*m*-Tol$^+$ *p*-Tol$^+$ Ben$^+$	29	19	3,400	540	100
PaW94(pGSH2224)	*m*-Tol$^-$ *p*-Tol$^+$ Ben$^+$	1	15	2,500	1,400	280

[a] See Fig. 2 for structures of plasmids.

[b] Expressed as nanomoles of substrate consumed per minute per milligram of protein. Data are taken from a typical experiment which gave the highest HMSH activity. C23O, Catechol 2,3-dioxygenase; 4OD, 4-oxalocrotonate decarboxylase; NE, not examined.

agarose or polyacrylamide, ligation by T4 ligase, and transformation have been described (24, 25).

Enzyme assays. Procedures for the assay of catechol 2,3-dioxygenase, HMSH, HMSD, 4OI, and 4-oxalocrotonate decarboxylase in cell-free extracts have been described (19). Kinetic constants for HMSD and HMSH were calculated from inverse plots of initial rates of dissimilation of substrates as a function of the substrate concentration.

RESULTS

4-OI is an essential enzyme of the 4-oxalocrotonate branch. Recent studies have shown that the *Hin*dIII A fragment of the TOL plasmid pWWO (Fig. 2) carries all of the *meta*-cleavage pathway enzymes structural genes, *xylXYZLEGFJKIH* (19, 20), plus *xylS*, the gene of the pathway positive regulator (17, 22, 41). Plasmid pMTO57 is a pBR322 derivative which carries this *Hin*dIII A segment and from which a collection of Tn*5* insertion mutants has been obtained (19). One of these mutant plasmids, pMTO57 *xylH310*::Tn*5*, containing a Tn*5* element within the structural gene of 4OI (*xylH*) which is located at the operator-distal end of the *meta*-cleavage operon, was chosen for the present study.

Strain PaW94 is a *P. putida* mutant that is defective in the chromosomally encoded benzoate dioxygenase (41) and is therefore unable to grow on benzoate. To permit the introduction into this strain of the partially functional *meta*-cleavage pathway of pMTO57 *xylH310*, the major *Hin*dIII fragment of this plasmid (i.e., lacking sequences downstream of the Tn*5* and thus lacking part of *xylH* and *xylS*; Fig. 2) was inserted into the pKT231 cloning vector at its unique *Hin*dIII site. The resulting plasmid, pNM1 (Fig. 2), was introduced by RP1-mediated mobilization into *P. putida* PaW94, followed by selection for streptomycin-resistant (Sm^r) transconjugants. None of the Sm^r transconjugants tested grew on minimal agar medium containing benzoate, *m*-toluate, or *p*-toluate as the sole carbon source (Ben$^-$ *m*-Tol$^-$ *p*-Tol$^-$ phenotype), as a result of the absence of *xylS*. Derivatives of strain PaW94(pNM1) which grew on *m*-toluate (*m*-Tol$^+$) could, however, be readily selected, although none of these could grow on *p*-toluate or benzoate. Two plasmids, designated pNM10 and pNM12, from two independent *m*-Tol$^+$ derivatives of PaW94(pNM1) were transformed into *E. coli* LE392 and subsequently introduced by RP1-mediated mobilization into plasmid-free *P. putida* PaW94. PaW94(pNM10) and PaW94(pNM12) transconjugants were also *m*-Tol$^+$ *p*-Tol$^-$ Ben$^-$, which indicates that the mutations responsible for *xylS*-independent growth on *m*-toluate reside on the transferred plasmids. Enzyme assays carried out on cell-free extracts of cultures of PaW94(pNM10) and PaW94(pNM12) grown in L broth revealed no 4OI activity, as expected, but high activities of other enzymes of the *meta*-cleavage pathway (Table 1). The mutations in pNM10 and pNM12 supplementary to the deletion in *xylH* created promoter activities which allowed *xylS*-independent expression of the *meta*-cleavage operon. From this experiment it may be concluded that 4-oxalocrotonate isomerase activity is essential for normal functioning of the oxalocrotonate branch.

Inactivation of the initial step in the 4-oxalocrotonate branch permits the catabolism of benzoate and *p*-toluate via the hydrolytic branch. Derivatives of *P. putida* strains PaW94(pNM10) and PaW94(pNM12) which were able to grow on *p*-toluate and benzoate could be readily selected. Plasmid DNA was isolated from *p*-Tol$^+$ Ben$^+$ derivatives of PaW94(pNM10) and PaW94(pNM12), transformed into *E. coli* LE392, and subsequently introduced by RP1-mediated mobilization into plasmid-free *P. putida* PaW94. Transconjugants were *m*-Tol$^+$ *p*-Tol$^+$ Ben$^+$. Therefore, the mutations responsible for the new growth phenotype are located on the plasmids derived from pNM10 and pNM12. Structural analysis of these derivatives, designated pNM100 and pNM120, respectively, showed that each had suffered a small deletion (less than 200 base pairs) in the region at which the *xylG* gene (codes for HMSD) was previously localized (19; Harayama, Rekik, and Timmis, manuscript in preparation; Fig. 2). Enzyme assays confirmed that the mutations had inactivated the *xylG* gene; cell-free extracts of cultures of strains PaW94(pNM100) and PaW94(pNM120) contained no significant HMSD activity (Table 1; the small values obtained in this assay are probably due to an inherently high background). Hence, the defect for growth on *p*-toluate and benzoate caused by the *xylH* mutation (defect in 4OI) could be suppressed by a mutation in the *xylG* gene (defect in HMSD).

Growth phenotype of *P. putida* bacteria containing an *xylG* deletion mutant plasmid. Because *xylH* mutants did not grow on benzoate and *p*-toluate, whereas the *xylH-xylG* double mutant did, it was of interest to determine the growth phenotype of the *xylG* single mutant. To do this, the *xylG* deletion mutation in pNM120(Δ*xylG120*) was transferred to TOL plasmid pWWO-161 by homologous recombination through the procedure outlined in Fig. 3. First, a 7-kilobase *Eco*RI segment of pNM120 containing Δ*xylG120* was subcloned in pACYC184 to form pGSH2751. The pGSH2751 plasmid, which is Tc^r, was transformed into *E. coli* LE392(pWWO-161), which is Ap^r as a result of the Tn*401* element carried by pWWO-161 (16), and Ap^r Tc^r transformants were selected. LE392(pWWO-161, pGSH2751) transformants were mated with *E. coli* ED2196, which is Nal^r, and Ap^r Tc^r Nal^r transconjugants were selected. Since the

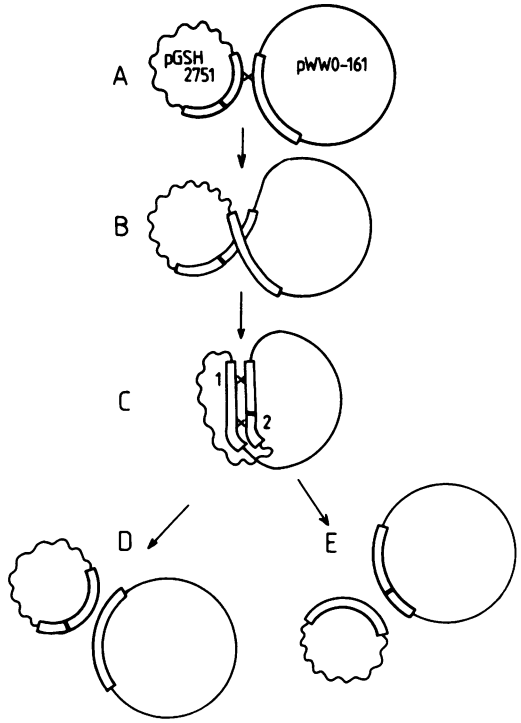

FIG. 3. Construction of *xylG* mutant of pWWO-161. (A) TOL plasmid pWWO-161 and pGSH2751. The pGSH2751 plasmid was constructed by cloning the 7.0-kilobase *Eco*RI fragment (▭) of pNM120 into pACYC184 (〰). This fragment ordinarily contains genes *xylYZLEGFJ*, but in this case *xylG* is the deletion derivative Δ*xylG120* (■). (B) Recombination at homologous TOL sequences on plasmids pWWO-161 and pGSH2751 produces a cointegrate. Transfer of Tc^r of pGSH2751 into a recipient can occur through this structure. (C) The B-form cointegrate is unstable in Rec$^+$ recipients and segregates into two plasmids by homologous recombination. Recombination at positions 1 and 2 produces structures D and E, respectively. Note that in E, Δ*xylG120* is transferred from pGSH2751 to pWWO-161. By mating *E. coli* cells containing plasmids in state D or E with *P. putida* PaW94, only pWWO-161 or its Δ*xylG120* derivative can be transferred to PaW94.

pACYC184 plasmid is neither conjugative nor mobilizable (9), transfer of pGSH2751 would most likely occur by transient formation of a cointegrate structure with pWWO-161, either by homologous recombination or by transposition of Tn*401*. If the cointegrate were formed by homologous recombination, it would readily resolve into two plasmids by reversal of the cointegration process. Such a recombinational event could exchange the two *xylG* loci on the pGSH2751 and pWWO-161 plasmids (homogenotization), resulting in a pWWO-161 derivative containing the Δ*xylG120* mutation (Fig. 3).

Four independent Ap^r Tc^r Nal^r transconjugants of *E. coli* ED2196 were mated with *P. putida* PaW94, and Cb^r transconjugants were selected on glucose-minimal medium plates. All *P. putida* transconjugants were Tc^s, indicating that the pWWO-161 plasmid, but not the pGSH2751 plasmid, was transferred into *P. putida* PaW94. Enzyme assays on extracts obtained from the transconjugants demonstrated that two of them were defective in *xylG*. Restriction endonuclease cleavage analysis of one *xylG* mutant of pWWO-161 confirmed that it had suffered a deletion corresponding in size and location to the previously characterized Δ*xylG120* mutation. Both *P. putida* strains containing XylG$^-$ mutant TOL plasmid grew as well on *m*- and *p*-toluates and benzoate as *P. putida* containing the *xylG*$^+$ TOL plasmid; the generation times of *P. putida* PaW94(pWWO-161) on benzoate and *m*- and *p*-toluates were 1.5, 1.2, and 1.9 h, respectively, whereas those of PaW94(pWWO-161 Δ*xylG120*) were 1.6, 1.2, and 2.4 h, respectively. The *xylG* mutants accumulated yellow compounds upon cultivation on benzoate and *p*-toluate.

Pathway branch selection is made by the enzyme HMSD. The fact that benzoate and *p*-toluate were not metabolized completely in the *xylH* mutant but were degraded via the hydrolytic branch when the first enzyme of the oxalocrotonate branch, HMSD, was defective suggested two alternative possibilities: (i) accumulation of 4-oxalocrotonate (compound V; R = R′ = H) or 2-hydroxy-5-methyl-hexa-2,4-diene-1,6-dioate (compound V; R = CH_3, R′ = H) is toxic to cells, or (ii) benzoate and *p*-toluate are metabolized in a normally functioning pathway, almost exclusively via the 4-oxalocrotonate branch.

To examine the toxicity of intermediates accumulated in *xylH* mutants, the effect of benzoate and *p*-toluate on growth of *P. putida* strains PaW94(pNM10) and PaW94(pNM12) with the permissive substrates glucose, *m*-toluate, and allylglycine was examined and compared with that of strains PaW94(pNM100) and PaW94(pNM120). Growth of PaW94(pNM10) and PaW94(pNM12) bacteria on plates containing allylglycine or *m*-toluate was slightly inhibited by *p*-toluate and significantly inhibited by benzoate. Such growth inhibition was not observed with PaW94(pNM100) and PaW94(pNM120) bacteria. Generation times of PaW94(pNM12) bacteria in glucose-minimal liquid media containing no aromatic compound, benzoate, *m*-toluate, and *p*-toluate were 1.0, 3.7, 0.9, and 2.9 h, respectively, whereas those of PaW94(pNM120) bacteria were 1.0, 1.2, 0.9, and 1.7 h, respectively. These results indicate that intermediates accumulated in *xylH* mutants during degradation of benzoate and *p*-toluate are somewhat toxic and reduce bacterial growth rates. This toxicity does not, however, completely prevent cell growth and thus does not explain why *xylH* mutants do not grow at all on benzoate and *p*-toluate. We therefore examined the second possibility, that the affinity of the dehydrogenase for ring cleavage products derived from benzoate and *p*-toluate greatly exceeds that of the hydrolase.

The concentration dependency of HMSD and HMSH activities with 2-hydroxymuconic semialdehyde (compound IV; R = R′ = H) was examined by using cell-free extracts of *P. putida* strains PaW94(pNM72) and PaW94(pNM12) (Fig. 4). Analysis of K_m and V_{max} demonstrated that the much higher activity of HMSD toward this ring-fission product at substrate concentrations lower than 20 μM was due to the lower K_m of HMSD (2 μM) compared with that of HMSH (>50 μM). The apparent K_m and V_{max} values of HMSD for 2-hydroxymuconic semialdehyde (compound IV; R = R′ = H), 2-hydroxy-5-methyl-hexa-2,4-diene-1,6-dioate (compound IV; R = CH_3, R′ = H), and 2-hydroxy-6-oxohepta-2,4-dienoate (compound IV; R = H, R′ = CH_3) were also examined by using cell-free extracts of *P. putida* PaW94 carrying pGSH2224, an *xylF* mutant of plasmid pNM72, to prevent simultaneous metabolism of these compounds by HMSH, which would have hindered accurate measurement of HMSD. Similarly, HMSH was analyzed in *P. putida* PaW94 carrying an *xylG* mutant plasmid, pNM120 (Fig. 5). As predicted, HMSD was found to have high affinity (K_m of 2 μM) for 2-hydroxymuconic semialdehyde (compound IV; R = R′ = H) and 2-hydroxy-5-methyl-hexa-2,4-diene-1,6-dioate (compound IV; R = CH_3, R′ = H), whereas HMSH

exhibited low affinity for these compounds (K_m of >50 μM) and moderate affinity for 2-hydroxy-6-oxohepta-hexa-2,4-dienoate (compound IV; R = H, R′ = CH_3) (K_m of 25 μM).

DISCUSSION

Several features of the functioning of the two branches of the TOL plasmid pWWO *meta*-cleavage pathway were clarified in the present study. First, *P. putida* bacteria containing a normal complement of the *meta*-cleavage enzymes were found to degrade *m*-toluate exclusively by the hydrolytic branch and benzoate and *p*-toluate almost exclusively via the 4-oxalocrotonate branch, as a result of the inability of HMSD to attack 2-hydroxy-6-oxohepta-2,4-dienoate (compound IV; R = H, R′ = CH_3; Fig. 1), the ring cleavage product derived from *m*-toluate, and of its high affinity for 2-hydroxymuconic semialdehyde (compound IV; R = R′ = H) and 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate (compound IV; R = CH_3, R′ = H), ring cleavage products derived from benzoate and *p*-toluate, respectively. Although hydroxymuconic semialdehyde hydrolase was found to metabolize 2-hydroxymuconic semialdehyde (compound IV; R = R′ = H) and 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate (compound IV; R = CH_3, R′ = H), its affinity toward these compounds was much less than that of HMSD. A moderate affinity toward these compounds of purified HMSH was recently reported (14). Therefore, at low substrate concentrations, these two semialdehydes are preferentially processed by HMSD. The inability of *xylH* mutants to grow on benzoate and *p*-toluate strongly suggests that the actual in vivo concentration of semialdehydes is so low as to prevent their processing by HMSH. Thus, suppression of the *xylH* mutation by a subsequent *xylG* mutation is understandable; a block in the 4-oxalocrotonate branch at the first (HMSD) rather than the second (4OI) step causes accumulation of semialdehydes; the resulting increase in their cellular concentration consequently permits their degradation by HMSH. The observation that the ring-fission product was accumulated in cultures of HMSD-negative mutants grown on benzoate or *p*-toluate supports this conclusion.

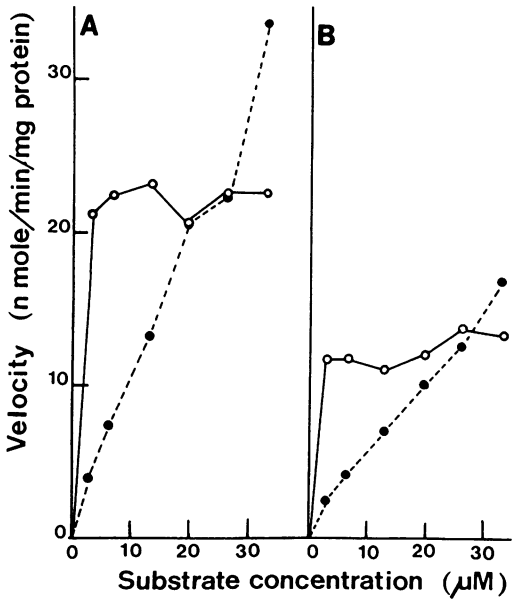

FIG. 4. Initial velocities of dissimilation of 2-hydroxymuconic semialdehyde by HMSH and HMSD as a function of substrate concentration. (A) HMSD (○) and HMSH (●) activities in *P. putida* PaW94(pNM12). (B) HMSD (○) and HMSH (●) activities in *P. putida* PaW94(pNM72). Values for HMSD and HMSH activities varied from experiment to experiment. At a 2-hydroxymuconic semialdehyde concentration of 33 μM, the activity of HMSD in PaW94(pNM12) and PaW94(pNM72) ranged from 22 to 80 and 11 to 34 nmol/min per mg of protein, respectively, whereas those for HMSH were 25 to 110 and 16 to 59 nmol/min per mg of protein, respectively. Despite these variations, the ratio of HMSH activity to HMSD activity at that concentration was rather constant at 1.4 ± 0.2.

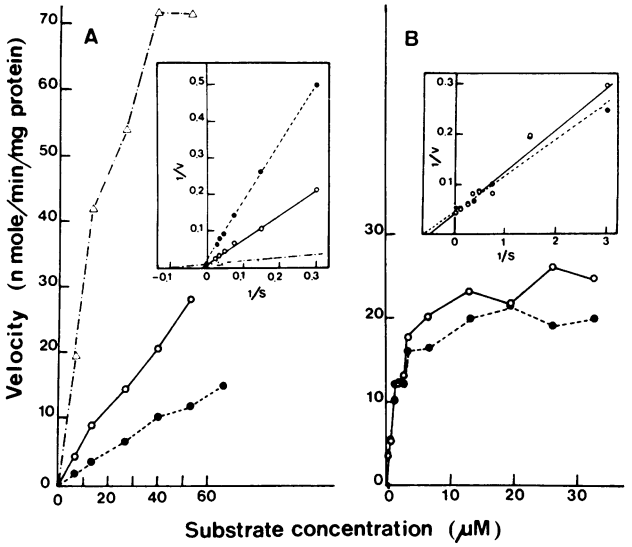

FIG. 5. Initial velocities of dissimilation of various ring cleavage products by HMSH or HMSD. (A) HMSH activity in *P. putida* PaW94(pNM120). (B) HMSD activity in PaW94(pGSH2224). Substrates were 2-hydroxymuconic semialdehyde (○), 2-hydroxy-5-methyl-hexa-2,4-diene-1,6-dioate (●), and 2-hydroxy-6-oxohepta-2,4-dienoate (△). The apparent K_m and V_{max} values were taken from inverse plots of initial velocities (v) to substrate concentrations (s) and assume single affinities and no negative cooperativity (see insets). K_m and V_{max} values of HMSH determined in A were as follows: K_m, 100, 100, and 25 μM; V_{max}, 80, 120, and 80 nmol/min per mg of protein for 2-hydroxymuconic semialdehyde, 2-hydroxy-5-methyl-hexa-2,4-diene-1,6-dioate, and 2-hydroxy-6-oxohepta-2,4-dienoate, respectively. Those of HMSD determined in B were as follows: K_m, 2 and 2 μM; V_{max}, 25 and 20 nmol/min per mg of protein for 2-hydroxymuconic semialdehyde and 2-hydroxy-5-methyl-hexa-2,4-diene-1,6-dioate, respectively.

Second, although the enol-keto interconversion of 4-oxalocrotonate occurs spontaneously in vitro (35), its rate in vitro must be too low to support cell growth; plasmids pNM10 and pNM12, which express constitutively all of the *meta*-cleavage pathway enzymes except 4OI, conferred upon *P. putida* PaW94 the ability to grow on *m*-toluate but not on *p*-toluate or benzoate. The growth defect of these *xylH* mutants (defective in the structural gene for 4OI) is most simply interpreted as signifying that 4OI is an enzyme essential for effective operation of 4-oxalocrotonate branch, through which ring fission products derived from benzoate and *p*-toluate are almost exclusively metabolized in vivo.

The physiological importance of an analogous isomerase in another aromatic degradation pathway has also been suggested. Bayly and his colleagues selected from *P. putida* U, which can degrade phenol and cresols, catabolic pathway mutants, one of which was defective in 4OI. This mutant was unable to grow on *p*-cresol and grew very slowly on phenol but normally on *m*- and *o*-cresols (38). Similarly, from strain P23X1, which can degrade 4-hydroxyphenylacetate, cata-

bolic mutants were isolated; one of these was found to be defective in 2-hydroxy-5-carboxymethyl-hexa-2,4-diene-1,6-dioate (compound V; R = CH_2COOH, R' = H) isomerase (3). They have concluded that the defect in these isomerases was responsible for the defect in the growth of the mutants, and hence that isomerases in these organisms are physiologically important. In the present study, a deletion mutation was introduced into the isomerase gene by site-directed mutagenesis, and the growth defect of the mutant was subsequently established; this avoided the possibility of leaky or multiple mutations and provided definitive proof for the earlier interpretations.

In contrast, our finding that the inactivation of TOL plasmid enzyme HMSD does not prevent growth on 4-methyl aromatic compounds differs from earlier results; the HMSD mutant of *P. putida* U grew normally on *o*- and *m*-cresol, slowly on phenol, and not at all on *p*-cresol (6). Although the hydrolase of this latter strain was found to metabolize the ring-fission products of catechol and 4-methylcatechol (compound IV; R = R' = H or R = CH_3 and R' = H; 4), its activity was lower than its activity toward the ring fission product of 3-methylcatechol (compound IV; R = H, R' = CH_3; 5); we concluded that this may be insufficient to support growth of cells on phenol and *p*-cresol. However, alternative interpretations are possible: first, since the HMSD mutant was isolated as a partial revertant of an HMSD-HMSH double mutant, HMSH in this revertant might not be fully functional; second, if the mutation in HMSD were leaky, the ring-fission compounds of catechol and 4-methylcatechol might not accumulate to a concentration sufficiently high for their effective metabolism by HMSH. To distinguish between the various possible interpretations, it would be desirable to introduce a defined deletion mutation into the HMSD genes of these latter bacteria and to analyze the phenotypes of the new mutants.

Although the physiological importance of 4OI is demonstrated beyond doubt, the precise reaction that it catalyzes is not established. If it would catalyze protonation of the α carbon of 2-hydroxy-hexa-2,4-diene-1,6-dioate (compound V; R = R' = H), the product would be the keto form of 4-oxalocrotonate (compound VIa), whereas if it would catalize protonation of the γ carbon, the product would be 2-oxohex-3-ene-1,6-dioate (compound VIb). Since the structure of compound VIII derived from 4-methylcatechol (R = CH_3, R' = H) has been determined to be 2-oxo-*cis*-hex-4-enoate (10), *cis-trans* isomerization should occur during transformation of compound IV to compound VIII (12, 38). If compound VIa is the real product, *cis-trans* isomerization should occur during conversion of compound VIa to compound VIII, whereas if compound VIb is an intermediate, isomerization of the $\alpha\beta$ unsaturated acid to the $\beta\gamma$ unsaturated acid should occur during transformation of compound VIb to compound VIII. Dagley and his colleague have emphasized that VIb is the more favorable structure for decarboxylation (12, 37).

Third, although *P. putida* PaW94 is defective for benzoate 1,2-dioxygenase, it contains a functionally intact, chromosomally encoded *ortho*-cleavage pathway which can metabolize catechol but not methyl-substituted catechols. *P. putida* PaW94 containing pNM10 and pNM12 should therefore be able to transform benzoate to catechol, by the plasmid-encoded toluate 1,2-dioxygenase and 1,2-dihydroxycyclohexa-3,5-diene-carboxylate dehydrogenase, and subsequently degrade catechol via the *ortho*-cleavage pathway. The Ben$^-$ character of these strains, however, indicated that synthesis of the *ortho*-cleavage pathway enzymes was not induced, almost certainly because the plasmid-encoded catechol 2,3-dioxygenase quickly dissimilated catechol and thereby prevented formation of *cis,cis*-muconate, the product of its *ortho* cleavage and the inducer of the *ortho*-cleavage pathway genes (20, 29).

Since *P. putida* PaW94 containing *xylG* (HMSD negative) mutants of TOL plasmid can grow on benzoate and *p*-toluate, the questions arise as to why the divergent branches exist and why a high-affinity hydrolase for *meta*-cleavage products of different catechols has not evolved? Metabolism of benzoate and *p*-toluate via the 4-oxalocrotonate branch produces NADH, whereas their metabolism via the hydrolytic branch produces formate but not NADH. NADH is produced by the further catabolism of formate by formate dehydrogenase, but to obtain the same energetic gain from the degradation of benzoate and *p*-toluate via the hydrolytic branch as is obtained from the 4-oxalocrotonate branch, coordinated synthesis of formate dehydrogenase is required. Therefore, one interesting possibility is that bacteria originally evolved only the 4-oxalocrotonate branch and that they subsequently developed the hydrolase in response to a need for extension of the substrate range of the pathway (36). Nevertheless, the low affinity of the hydrolase for 2-hydroxymuconic semialdehyde (compound IV; R = R' = H) and 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate (compound IV; R = CH_3 R = H) may have been retained so that these compounds would be channeled into the energetically favorable 4-oxalocrotonate branch. The natural occurrence of bacteria which express only HMSD activity (35) suggests that such ancestral pathways exist in some bacterial species.

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LITERATURE CITED

1. **Adelberg, E. A., M. Mandel, and G. C. C. Chen.** 1965. Optimal condition for mutagenesis by N-methyl-N'-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. **Bagdasarian, M., R. Lurz, B. Rückert, F. C. H. Franklin, M. M. Bagdasarian, J. Frey, and K. N. Timmis.** 1981. Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. Gene **16**:237–247.
3. **Barbour, M. G., and R. C. Bayly.** 1980. Mutants defective in isomerase and decarboxylase activities of the 4-hydroxyphenylacetic acid *meta*-cleavage pathway in *Pseudomonas putida*. J. Bacteriol. **142**:480–485.
4. **Bayly, R. C., and S. Dagley.** 1969. Oxoenoic acids as metabolites in the bacterial degradation of catechols. Biochem. J. **111**: 303–307.
5. **Bayly, R. C., and D. Di Berardino.** 1978. Purification and properties of 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase from two strains of *Pseudomonas putida*. J. Bacteriol. **134**: 30–37.
6. **Bayly, R. C., and G. J. Wigmore.** 1973. Metabolism of phenol and cresols by mutants of *Pseudomonas putida*. J. Bacteriol. **113**:1112–1120.
7. **Cain, R. B., and D. R. Farr.** 1968. Metabolism of arylsulphonates by microorganisms. Biochem. J. **106**:859–877.
8. **Catterall, F. A., J. M. Sala-Trepart, and P. A. Williams.** 1971. The coexistence of two pathways for the metabolism of 2-hydroxymuconic semialdehyde in a naphthalene-grown *Pseudomonas*. Biochem. Biophys. Res. Commun. **43**:463–469.
9. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmids. J. Bacteriol. **134**:1141–1156.

10. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. USA **62**:1159–1166.
11. **Collinsworth, W. L., P. J. Chapman, and S. Dagley.** 1973. Stereospecific enzymes in the degradation of aromatic compounds by *Pseudomonas putida*. J. Bacteriol. **113**:922–931.
12. **Dagley, S.** 1978. Pathways for the utilization of organic growth substrates, p. 305–388. *In* L. N. Ornston and J. R. Sokatch (ed.), The bacteria, vol. 6. Academic Press, Inc., New York.
13. **Dagley, S., and D. T. Gibson.** 1965. The bacterial degradation of catechol. Biochem. J. **95**:466–474.
14. **Duggleby, C. J., and P. A. Williams.** 1986. Purification and some properties of the 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase (2-hydroxymuconic semialdehyde hydrolase) encoded by the TOL plasmid pWWO from *Pseudomonas putida* mt-2. J. Gen. Microbiol. **132**:717–726.
15. **Feist, C. F., and G. D. Hegeman.** 1969. Phenol and benzoate metabolism by *Pseudomonas putida*: regulation of tangential pathways. J. Bacteriol. **100**:869–877.
16. **Franklin, F. C. H., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis.** 1981. Molecular and functional analysis of the TOL plasmid pWWO from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring *meta* cleavage pathway. Proc. Natl. Acad. Sci. USA **78**:7458–7462.
17. **Franklin, F. C. H., P. R. Lehrbach, R. Lurz, B. Rückert, M. Bagdasarian, and K. N. Timmis.** 1983. Locarization and functional analysis of transposon mutations in regulatory genes of the TOL catabolic pathway. J. Bacteriol. **154**:676–685.
18. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.
19. **Harayama, S., P. R. Lehrbach, and K. N. Timmis.** 1984. Transposon mutagenesis analysis of *meta*-cleavage pathway operon genes of the TOL plasmid of *Pseudomonas putida* mt-2. J. Bacteriol. **160**:251–255.
20. **Harayama, S., M. Rekik, and K. N. Timmis.** 1986. Genetic analysis of a relaxed substrate specificity aromatic ring dioxygenase, toluate 1,2-dioxygenase, encoded by TOL plasmid pWWO of *Pseudomonas putida*. Mol. Gen. Genet. **202**:226–234.
21. **Harwood, C. S., and L. N. Ornston.** 1984. TOL plasmid can prevent induction of chemotactic responses to aromatic acids. J. Bacteriol. **160**:797–800.
22. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1981. Molecular cloning of gene *xylS* of the TOL plasmid: evidence for positive regulation of the *xylDEGF* operon by *xylS*. J. Bacteriol. **148**:413–418.
23. **Johnson, B. F., and R. Y. Stanier.** 1971. Dissimilation of aromatic compounds by *Alcaligenes entrophus*. J. Bacteriol. **107**:468–475.
24. **Lehrbach, P. R., J. Zeyer, W. Reineke, W.-J. Knachmuss, and K. N. Timmis.** 1984. Enzyme recruitment in vitro: use of cloned genes to extend the range of holoaromatics degraded by *Pseudomonas* sp. strain B13. J. Bacteriol. **158**:1025–1032.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbor, N.Y.
26. **Mermod, N., P. R. Lehrbach, W. Reineke, and K. N. Timmis.** 1984. Transcription of the TOL plasmid toluate catabolic pathway operon of *Pseudomonas putida* is determined by a pair of co-ordinately and positively regulated overlapping promoters. EMBO J. **3**:2461–2466.
27. **Murray, K., C. J. Duggleby, J. M. Sala-Trepat, and P. A. Williams.** 1972. The metabolism of benzoate and methylbenzoates *via* the *meta*-cleavage pathway by *Pseudomonas arvilla* mt-2. Eur. J. Biochem. **28**:301–310.
28. **Nakazawa, T., and T. Yokota.** 1973. Benzoate metabolism in *Pseudomonas putida* (*arvilla*) mt-2: demonstration of two benzoate pathways. J. Bacteriol. **115**:262–267.
29. **Nishizuka, Y., A. Ichiyama, S. Nakamura, and O. Hayaishi.** 1962. A new metabolic pathway of catechol. J. Biol. Chem. **237**:PC268–PC272.
30. **Ornston, L. N.** 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. IV. Regulation. J. Biol. Chem. **241**:3800–3810.
31. **Ornston, L. N., and W.-K. Yeh.** 1982. Reccurring themes and repeated sequences in metabolic evolution, p. 105–126. *In* A. M. Chakrabarty (ed.), Biodegradation and detoxification of environmental pollutants. CRC Press, Boca Raton, Fla.
32. **Reineke, W., D. J. Jeenes, P. A. Williams, and H.-J. Knackmuss.** 1982. TOL plasmid pWWO in constructed halobenzoate-degrading *Pseudomonas* strains: prevention of *meta* pathway. J. Bacteriol. **150**:195–201.
33. **Ribbons, D. W.** 1970. Specificity of monohydric phenol oxidations by *meta* cleavage pathways in *Pseudomonas aeruginosa* T1. Arch. Mikrobiol. **74**:103–115.
34. **Ribbons, D. W., and R. W. Eaton.** 1982. Chemical transformations of aromatic hydrocarbons that support the growth of microorganisms, p. 59–84. *In* A. M. Chakrabarty (ed.), Biodegradation and detoxification of environmntal pollutants. CRC Press, Boca Raton, Fla.
35. **Sala-Trepat, J. M., and W. C. Evans.** 1971. The *meta* cleavage of catechol by *Azotobacter* species. 4-Oxalocrotonate pathway. Eur. J. Biochem. **20**:400–413.
36. **Sala-Trepat, J. M., K. Murray, and P. A. Williams.** 1972. The metabolic devergence in the *meta* cleavage of catechols by *Pseudomonas putida* NCIB 10015. Physiological significance and evolutionary implications. Eur. J. Biochem. **28**:347–356.
37. **Sparnins, V. L., P. J. Chapman, and S. Dagley.** 1974. Bacterial degradation of 4-hydroxyphenylacetic acid and homoprotocatechuic acid. J. Bacteriol. **120**:159–167.
38. **Wigmore, G. J., R. C. Bayly, and D. Di Berardino.** 1974. *Pseudomonas putida* mutants defective in the metabolism of the products of *meta* fission of catechol and its methyl analogs. J. Bacteriol. **120**:31–37.
39. **Williams, P. A., and K. Murray.** 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. **120**:416–423.
40. **Wong, C. L., and N. W. Dunn.** 1974. Transmissible plasmid coding for the degradation of benzoate and *m*-toluate in *Pseudomonas arvilla* mt-2. Genet. Res. **23**:227–232.
41. **Worsey, M. J., F. C. H. Franklin, and P. A. Williams.** 1978. Regulation of the degradative pathway enzymes coded for by the TOL plasmid (pWWO) from *Pseudomonas putida* mt-2. J. Bacteriol. **134**:757–764.
42. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. **124**:7–13.
43. **Yen, K.-M., and I. C. Gunsalus.** 1982. Plasmid gene organization: naphthalene salicylate oxidation. Proc. Natl. Acad. Sci. USA **79**:874–878.