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Control of *meta*-Cleavage Degradation of 4-Hydroxyphenylacetate in *Pseudomonas putida*

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Synthesis of enzymes of the 4-hydroxyphenylacetate *meta*-cleavage pathway was studied in *Pseudomonas putida* wild-type strain P23X1 (NCIB 9865) and mutant strains which had either structural or regulatory gene mutations. Induction studies with mutant strains each defective in an enzyme of the pathway showed that 4-hydroxyphenylacetate induced the hydroxylase and that 3,4-dihydroxyphenylacetate induced the 2,3-oxygenase, aldehyde dehydrogenase, isomerase, decarboxylase, and hydratase. This showed that the hydroxylase structural gene does not exist in an operon that contains any other structural gene of this *meta* pathway. Studies of mutant strains that synthesized constitutively the 2,3-oxygenase and subsequent enzymes suggested that the regulation of synthesis of these enzymes was coincident, and, in such strains, the hydroxylase was inducible only. Observations made with a putative polarity mutant that lacked 2,3-oxygenase activity suggested that the structural genes encoding this enzyme and subsequent enzymes of the pathway exist in the same operon. Studies of a regulatory mutant strain that was defective in the induction of the 2,3-oxygenase and subsequent enzymes suggest that the 2,3-oxygenase operon is under positive control.

Studies of the mechanisms which regulate the synthesis of enzymes involved in the degradation of aromatic compounds by *meta*-cleavage pathways have been confined to systems in which catechol, or its 3- or 4-methyl derivatives, are substrates for ring cleavage. Models have been proposed for the regulation of the plasmid-encoded enzymes which catabolize toluene, *m*-xylene, and *p*-xylene in *Pseudomonas putida* strains mt-2 (17) and MT-20 (15, 18) and the apparently chromosomally encoded enzymes for the degradation of phenol and the isomers of cresol by *P. putida* strain U (14).

P. putida strain P23X1 (NCIB 9867) degrades 4-hydroxyphenylacetate (4-HPA) by a *meta*-cleavage pathway (Fig. 1) elucidated in two strains of *P. putida* and an *Acinetobacter* sp. by Sparnins et al. (13). In an earlier communication (2), it was shown that growth of P23X1 on 4-HPA resulted in synthesis of all enzymes of the degradative pathway, and we presented evidence that the genes encoding the first four enzymes of the pathway shown in Fig. 1 appeared to exist as two operons. The hydroxylase was induced only by 4-HPA, and the 2,3-oxygenase and aldehyde dehydrogenase were induced by 3,4-dihydroxyphenylacetate (3,4-DHPA). This paper reports a study of some regulatory and structural mutant strains defective in enzymes B to F (Fig. 1) of the 4-HPA *meta*-cleavage pathway.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains and procedures used in mutant isolation are listed in Table 1.

Media and culture conditions. Conditions of growth, induction of enzymes, and preparation of cell suspensions were described previously (2, 4). Preparation of cell extracts was as described by Barbour and Bayly (2), and protein concentration was determined by the method of Lowry et al. (8). Solid media were prepared by the addition of Difco agar (1.5% [wt/vol]) to basal medium containing the required carbon source. When 3,4-DHPA was added to solid medium, a dark brown color formed in the medium within 12 h. This was possibly formed by auto-oxidation of 3,4-DHPA to a quinone-type compound similar to that formed by 3-methylcatechol and 4-methylcatechol (11). When sodium thioglycolate (1% [wt/vol]) was incorporated in the medium, the dark brown product did not form until about 72 h after the addition of 3,4-DHPA, and so, for routine use, solid media containing 3,4-DHPA also contained 1% sodium thioglycolate. Neither the rate nor extent of growth of *P. putida* P23X1 was affected by this addition.

Preparation of heat-treated extract. Crude extracts of *P. putida* P23X1, grown on fumarate in the presence of 2.5 mM 4-HPA, were heated in a water bath at 50°C for 3 min and immediately chilled on ice, and the precipitate was removed by centrifugation.

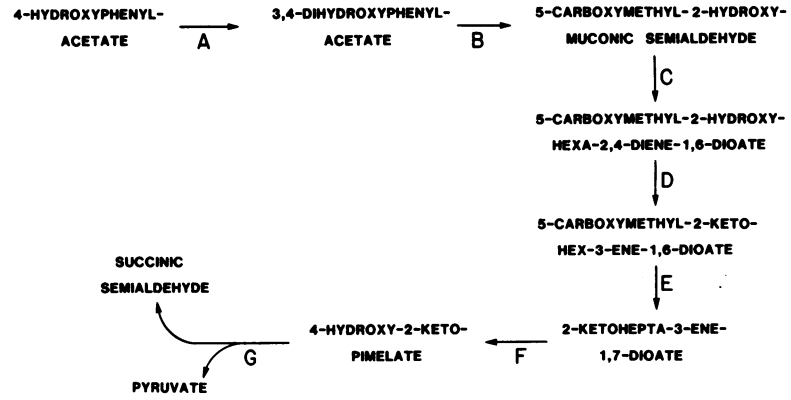

FIG. 1. *The meta-cleavage pathway for the degradation of 4-hydroxyphenylacetate by P. putida. A, Hydroxylase; B, 2,3-oxygenase; C, aldehyde dehydrogenase; D, isomerase; E, decarboxylase; F, hydratase, G, aldolase.*

TABLE 1. *P. putida strains used*

Strain	Relevant characteristic	Method of isolation	Reference
P23X1 (NCIB 9865)	Wild type		2,3
P23X2	Putative polarity mutant	Antibiotic contraselection[a]	This study
P23X6	Hydroxylase defective	Antibiotic contraselection[a]	2
P23X16	Isomerase defective	As P23X2	3
P23X19	Decarboxylase defective	As P23X2	3
P23X21	Dehydrogenase defective	As P23X6	2
P23X28	2,3-Oxygenase defective	As P23X6	2
P23X29	Regulatory mutant	As P23X2	This study
P23XCI	Constitutive mutant	Chemostat	This study
P23XC5	Constitutive mutant	Alternate subculture	This study
P23XC7	Constitutive mutant	As P23XC5	This study
P23X29-R2	Revertant of P23X29	Growth on 4-HPA	This study
P23X29-R3	Revertant of P23X29	Growth on 3,4-DHPA	This study

[a] Contraselective carbon source was 3,4-DHPA, and selective carbon source was quinate.
[b] Contraselective carbon source was 4-HPA, and selective carbon source was quinate.

This treatment abolished dehydrogenase activity but did not reduce 2,3-oxygenase activity.

Isolation and selection of mutant strains. Methods used for the isolation of mutant strains unable to grow at the expense of 4-HPA or 3,4-DHPA were described previously by Barbour and Bayly (2, 3).

Isolation of 2,3-oxygenase-constitutive mutants. The following two methods were used.

(i) Chemostat. This method was based on the method of Horiuchi et al. (7), and the chemostat was similar to that described by Baker (1). The volume of the culture was 250 ml in a 500-ml vessel, and aeration was by passage of air (30 liters h^{-1}) over the surface of the stirred culture (300 rpm). The culture was grown under batch conditions in basal medium containing 1 mM 3,4-DHPA to an optical density of approximately 0.1 at 580 nm when addition of medium commenced, and incubation was continued for 260 h. Medium for addition was kept saturated with nitrogen to retard auto-oxidation of 3,4-DHPA. A sample of the culture was then diluted, plated onto basal medium containing 10 mM fumarate, and, after 48 h of incubation, plates containing 50 to 300 discrete colonies were sprayed with an aqueous solution of 3,4-DHPA (10 mM) and chloramphenicol (100 $\mu g\ ml^{-1}$). Chloramphenicol was used to inhibit synthesis of 2,3-oxygenase that might have been induced by 3,4-DHPA. All colonies turned yellow within 2 min due to the formation of 5-carboxymethyl-2-hydroxymuconic semialdehyde and were presumed to be constitutive for 2,3-oxygenase. To avoid selection of siblings, only one isolate from the chemostat culture was retained for further study.

(ii) Alternate subculture. This method was based upon that described by Parke and Ornston (10). *P. putida* P23X1 was subcultured alternately between basal medium that contained either 2.5 mM 3,4-DHPA or 10 mM fumarate, the cultures being incubated overnight with shaking. After 10 such cycles, the cultures were screened as described above for the chemostat method. Only one isolate was retained from each series of cultures.

Enzyme assays. Methods for preparation of substrates for the assays of (i) dehydrogenase and hydratase were described by Sparnins et al. (13), and (ii) isomerase and decarboxylase were described by Barbour and Bayly (3). Assays of enzyme activities were described previously (2, 3). Results obtained for the

assay of specific activity of the hydratase were low and not reproducible, and, therefore, hydratase activity was designated as either present (+) or not detectable (−). Assays for aldolase activity were not carried out.

Determination of 3,4-DHPA. 3,4-DHPA in liquid media was determined by the amount of 5-carboxymethyl-2-hydroxymuconic semialdhyde produced after the addition of heat-treated extract to the sample to be tested. The reaction mixture contained, in a total volume of 3 ml, 0.1 M phosphate buffer (pH 7.4), an aliquot of the sample to be tested, and heat-treated extract (about 1 mg of protein). The reaction was monitored at 380 nm. When the concentration of 3,4-DHPA in the sample exceeded 0.05 μmol ml^{-1}, the reaction did not go to completion, and such samples were diluted with 0.1 M phosphate buffer (pH 7.4) to within the range of 0.003 to 0.05 μmol ml^{-1}.

RESULTS

When *P. putida* P23X1 was grown either on 3,4-DHPA or on fumarate in the presence of 3,4-DHPA, the following enzymes shown in Fig. 1 were detected: 2,3-oxygenase, dehydrogenase, isomerase, decarboxylase, and hydratase. No activities of these enzymes were detected when growth was in the absence of 3,4-DHPA. When each of the mutant strains P23X28, P23X21, P23X16, and P23X19 was grown on fumarate in the presence of 3,4-DHPA, all of the above enzymes were detected in each strain except for 2,3-oxygenase in P23X28, dehydrogenase in P23X21, isomerase in P23X16, and decarboxylase in P23X19 (Table 2). Therefore 2,3-oxygenase and the subsequent enzymes of the pathway were induced coincidently by 3,4-DHPA.

Induction specificities of enzymes of the pathway appeared to be strict because, of the compounds tested, only 4-HPA induced the hydroxylase, and only 3,4-DHPA induced the other enzymes. The following compounds failed to induce any enzymes of the following pathway: 2-hydroxyphenylacetate, 3-hydroxyphenylacetate, 4-hydroxybenzoate, *p*-cresol, 4-hydroxyphenylpropionate, 3,4-dihydroxybenzoate, 3,4-dihydroxybenzaldehyde, 3,4-dihydroxycinnamate, 3,4-dihydroxyphenylalanine, 3,4-dimethoxyphenylacetate, 3,4-dimethoxycinnamate, 3,5-dimethoxy-4-hydroxycinnamate, 3-hydroxy-4-methylbenzoate, 4-hydroxy-3-methylbenzoate, 4-hydroxy-3-methoxybenzoate, 4-hydroxy-3-methoxyphenylacetate, catechol, and 3- and 4-methylcatechol. Substrate specificities of the enzymes of the 4-HPA *meta*-cleavage pathway were strict because none of the above compounds served as a substrate when tested for oxygen uptake with cells of *P. putida* P23X1 grown on either 4-HPA or 3,4-DHPA, and none of the enzymes C to F (Fig. 1) had activity against the intermediates for the analogous reactions (4) in the phenol-*p*-cresol *meta*-degradation pathway in strain P23X1 (unpublished data).

TABLE 2. *Specific activities of enzymes of the 4-HPA meta-cleavage pathway in crude extracts of P. putida P23X1 and mutants P23X28, P23X21, P23X16, and P23X19 grown in the presence of 3,4-DHPA*

Enzyme	Sp act[a] for following strain:				
	P23X1	P23X28	P23X21	P23X16	P23X19
2,3-Oxygenase	0.950	<0.001	0.242	0.510	0.290
Dehydrogenase	0.070	0.034	<0.001	0.045	0.037
Isomerase[b]	0.105	0.062	0.058	<0.001	0.059
Decarboxylase[c]	0.026	0.020	0.013	0.020	<0.001
Hydratase	+[d]	+	+	+	+

[a] Expressed as micromoles of product formed or substrate removed per minute per milligram of protein.

[b] Corrected for nonenzymatic breakdown of substrate.

[c] Expressed as micromoles of substrate used per milligram of protein after 1 min.

[d] +, Activity detected.

Constitutive mutant strains. 2,3-Oxygenase and subsequent enzymes were detected in crude extracts of mutants P23XC1, P23XC5, and P23XC7 grown in fumarate medium (Table 3). Only with mutant strain P23XC1 were the activities of enzymes B to F increased during growth on fumarate in the presence of 3,4-DHPA. Hydroxylase activity was not detected when these mutants were grown on fumarate, but when the mutants were grown in the presence of 4-HPA, hydroxylase activity was detected at wild-type-induced levels.

Putative polarity mutant. When mutant P23X2 was grown on fumarate in the presence of 3,4-DHPA, no 2,3-oxygenase activity was detected but enzymes C to F were detected at about 5 to 10% of wild-type-induced levels. Mutant strain P23X2 reverted to growth on 3,4-DHPA at a frequency of appearance of about 10^{-7} per cell plated, and such revertants were indistinguishable phenotypically and by enzyme assay from the wild type.

No hydroxylase activity was detected in cells of *P. putida* P23X2 grown on fumarate in the presence of 4-HPA, but 3,4-DHPA accumulated in the medium of such cultures (Fig. 2), indicating the presence of an active hydroxylase. Similar observations were made with the 2,3-oxygenase-defective mutant P23X28. At 2.5 h after the addition of 4-HPA, the concentration of 3,4-DHPA was at a maximum, and, at this time, the culture was dark brown, due possibly to the formation of a quinone-like compound mentioned earlier. On further incubation, the concentration of 3,4-DHPA decreased (Fig. 2), but because the mutant was defective in 2,3-oxygenase activity (Table 2), the decrease was assumed

TABLE 3. *Specific activities of enzymes of the 4-HPA meta-cleavage pathway in crude extracts of P. putida P23X1 and mutants P23XC1, P23XC5, and P23XC7 grown on fumarate or in the presence of 3,4-DHPA*

Enzyme	Sp act[a] for following strain:						
	P23X1[b]	P23XC1		P23XC5		P23XC7	
	3,4-DHPA	Fumarate	3,4-DHPA	Fumarate	3,4-DHPA	Fumarate	3,4-DHPA
2,3-Oxygenase	0.852	0.373	0.617	0.880	0.840	0.480	0.490
Dehydrogenase	0.074	0.055	0.079	0.079	0.077	0.056	0.058
Isomerase[c]	0.152	0.066	0.106	0.192	0.188	0.082	0.084
Decarboxylase[d]	0.025	0.010	0.020	0.021	0.023	0.016	0.016
Hydratase	+[e]	+	+	+	+	+	+

[a] Expressed as micromoles of product formed or substrate removed per minute per milligram of protein.
[b] No activities were detected when strain P23X1 was grown on fumarate.
[c] Corrected for nonenzymatic breakdown of substrate.
[d] Expressed as micromoles of substrate used per milligram of protein after 1 min.
[e] +, Activity detected.

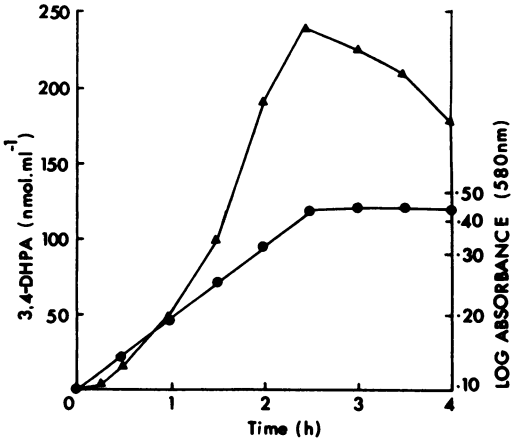

FIG. 2. *Accumulation of 3,4-DHPA in the culture supernatant fluid of P. putida mutant strain P23X2 grown in the presence of 2.5 mM 4-HPA. ▲, Concentration of 3,4-DHPA; ●, absorbance at 580 nm. Strain P23X2 was grown in fumarate until the absorbance at 580 nm was approximately 0.1, when 4-HPA was added to a final concentration of 2.5 mM, and incubation was continued for 4 h. The time shown is after the addition of 4-HPA.*

to be due to nonenzymatic oxidation of 3,4-DHPA. Neither the dark brown product that was formed in the culture nor that formed when a solution of 3,4-DHPA was held at 30°C for 2 h was a substrate of 2,3-oxygenase (Barbour, unpublished data). 3,4-DHPA was not detected in cultures of either strain P23X1 or the hydroxylase-defective strain P23X6 grown under the same conditions as indicated in Fig. 2. It is not known why it was not possible to detect hydroxylase activity in *P. putida* P23X2 grown in the presence of 4-HPA.

It was assumed, therefore, that the mutation in strain P23X2 occurred within the 2,3-oxygenase structural gene, and, because of the apparent coordinate reduction in the levels of enzymes C to F, it was proposed that the mutation was polar.

Regulatory mutant defective in induction of all enzymes of the pathway. Mutant P23X29 grew more slowly than P23X1 when either 4-HPA or 3,4-DHPA was the sole source of carbon, with doubling times of 174 and 196 min, respectively, compared with 75 and 78 min, respectively, for strain P23X1. Revertants of strain P23X29 appeared at a frequency of about 10^{-8} per cells plated on either 4-HPA or 3,4-DHPA. It was assumed, therefore, that strain P23X29 arose as a result of a single-point mutation. All revertants isolated grew at wild-type rates on both substrates. After growth of strain P23X29 with either 4-HPA or 3,4-DHPA, all enzymes of the pathway were detected at about 30% of wild-type-induced levels, but no activities of these enzymes were detected in fumarate-grown cells (Table 4). The inability to detect hydroxylase activity by measurement of oxygen uptake by strain P23X2 makes it uncertain whether the low hydroxylase activity in strain P23X29 was a consequence of the mutation.

Strains P23X29-R2 and P23X29-R3, revertants of mutant P23X29, were selected for wild-type rate of growth on 4-HPA and 3,4-DHPA, respectively. After growth of these revertants with either 4-HPA or 3,4-DHPA, enzymes A to F were detected at about wild-type-induced levels (Table 4). Constitutive syntheses of enzymes B to E were detected at about 2 to 8% of the 3,4-DHPA-induced levels. Constitutive synthesis of neither the hydroxylase nor the hydratase was detected. If, as will be proposed, the hydratase structural gene existed in the same operon as that of the 2,3-oxygenase, then the inability to detect constitutive hydratase activity at the same levels of enzymes B to E was not unexpected because of the difficulty in the de-

TABLE 4. *Specific activities of enzymes of the 4-HPA meta-cleavage pathway in P. putida P23X1, P23X29, and P23X29-R2 grown on fumarate or in the presence of either 4-HPA or 3,4-DHPA*

Enzyme	Sp act[a] for following strain:						
	P23X1[b]		P23X29[b]		P23X29-R2[c]		
	4-HPA	3,4-DHPA	4-HPA	3,4-DHPA	Fumarate	4-HPA	3,4-DHPA
Hydroxylase[d]	388	<5	144	<5	<5	516	<5
2,3-Oxygenase	0.990	0.310	0.350	0.080	0.015	0.870	0.780
Dehydrogenase	0.068	0.052	0.023	0.015	0.003	0.058	0.064
Isomerase[e]	0.115	0.097	0.042	0.026	0.007	0.097	0.084
Decarboxylase[f]	0.026	0.028	0.007	0.011	0.002	0.036	0.027
Hydratase	+[g]	+	+	+	−	+	+

[a] Expressed as micromoles of substrate used or product formed per minute per milligram of protein.
[b] No activities were detected when either strain P23X1 or P23X29 was grown on fumarate.
[c] Strain P23X29-R3 behaved similarly to strain P23X29-R2.
[d] Values, corrected for endogenous respiration, are expressed as microliters of oxygen taken up per hour per milligram (dry weight) of cells.
[e] Corrected for nonenzymatic breakdown of substrate.
[f] Expressed as micromoles of substrate used per milligram of protein after 1 min.
[g] +, Activity detected; −, activity not detected.

tection of low hydratase activities. These results suggested that in strain P23X29 a mutation occurred within a gene that controlled the expression of enzymes B to F of the pathway.

Mutant P23X29 grew at wild-type rate when phenol, *o*-, *m*-, and *p*-cresol, quinate, or benzoate was the sole source of carbon, indicating that reduced synthesis of enzymes of the 4-HPA *meta*-cleavage pathway was not due to a mutation in some regulatory mechanism that controlled a number of degradative pathways.

DISCUSSION

Results showed that enzymes of the 4-HPA *meta*-cleavage pathway in *P. putida* P23X1 were induced after two sequential inductive events: 4-HPA induced the hydroxylase, and 3,4-DHPA induced the 2,3-oxygenase and subsequent enzymes. This mode of induction differed from that of the catechol *meta*-cleavage pathway used for the degradation of phenol in *P. putida* strain U (14) and *P. aeruginosa* strain T1 (11) and of toluene in *P. putida* strains mt-2 (14) and MT20 (15, 18), wherein all enzymes of the pathway were induced by the primary substrate. The 4-HPA and catechol *meta*-cleavage pathways differ in at least two other aspects. Enzymes of the catechol *meta*-cleavage pathway were of broad substrate specificity and were induced by a number of different compounds, whereas both induction and substrate specificity of enzymes of the 4-HPA *meta*-cleavage pathway appeared to be strict.

Studies with blocked mutants showed that enzymes B to F (Fig. 1) were induced coincidently by 3,4-DHPA. Conicident synthesis of these enzymes was confirmed by the finding that mutant strains P23XC1, P23XC5, and P23XC7 synthesized constitutively the 2,3-oxygenase and subsequent enzymes. In the putative polarity mutant P23X2, 2,3-oxygenase was not detected, and the levels of the dehydrogenase and subsequent enzymes were reduced coordinately to about 5 to 10% of wild-type-induced levels. It was considered likely that the lesion within the 2,3-oxygenase structural gene in mutant P23X2 was a polar mutation suggesting that the genes encoding the 2,3-oxygenase and subsequent enzymes existed in the same operon (2,3-oxygenase operon) and were transcribed into a polycistronic message. The existence of a polar mutation indicates the direction in which the operon is transcribed and translated (19); therefore, the 2,3-oxygenase structural gene would be situated promoter-proximal within the operon, and the other structural genes would be promoter-distal from the 2,3-oxygenase structural gene. An order cannot yet be ascribed to the genes encoding the dehydrogenase and subsequent enzymes. Observations of constitutive synthesis of enzymes B to F in mutants P23XC1, P23XC5, and P23XC7 suggest that the mutation in each strain must have occurred within a regulatory gene that controlled the expression of the 2,3-oxygenase operon. It is not possible yet to identify these mutated loci due to the lack of a suitable system of genetic analysis.

It is not understood why it was not possible to detect hydroxylase activity in cells of strain P23X2 grown on fumarate in the presence of 4-HPA, although the results showed that the hy-

droxylase was functional because there was formation of 3,4-DHPA. For this reason, no conclusion has been drawn as to any relationship between the regulation of synthesis of the hydroxylase and that of the other pathway enzymes.

Observations made with mutant P23X29 suggested that a mutation affected the function of a regulatory molecule, resulting in decreased efficiency of induction by 3,4-DHPA of the 2,3-oxygenase operon. Since revertants of mutant P23X29 had low constitutive levels of 2,3-oxygenase and subsequent enzymes, it is possible that the 2,3-oxygenase operon is under positive control. Mutant P23X29 showed only some of the characteristics of a positive regulator non-inducible mutation because its revertants showed only partial constitutivity. Revertants of mutants defective in induction of enzymes of the positively controlled arabinose (12), amidase (5), and toluene (15, 17) systems have been either inducible, constitutive, or partially constitutive. If control was negative, revertants of such "non-inducible" mutants would be expected to show a "high" constitutivity phenotype (16). For the reasons already given, it was not possible to determine whether the expression of the hydroxylase structural gene was affected also by the mutation in strain P23X29. The observation of low constitutive expression of the 2,3-oxygenase operon suggested that the mutation in strain P23X29 did not occur within an initiator-promoter region of the operon because it was considered unlikely that revertants of such initiator-promoter mutants would show low-level constitutivity. The phenotype of strain P23X29 could have arisen also if a permease that functioned to transport inducing molecules into the cell had been altered by mutation. This possibility was considered unlikely because revertants of such a permease mutant would not be expected to show partial constitutivity of any pathway enzyme as found in strains P23X29-R2 and P23X29-R3. Worsey et al. (17) and Williams and Worsey (15) proposed positive control as the mode of regulation of toluene degradation in two strains of *P. putida*, but Wigmore et al. (14) suggested that, in phenol degradation by *P. putida* strain U, the hydroxylase structural gene was under positive control, and the 2,3-oxygenase operon was under negative control. Recent reports from Franklin and Williams (6) and Nakazawa et al. (9) have produced further evidence to support the proposal that the two regulons of the pathway for the degradation of toluene by *P. putida* mt-2 are under positive control. Genetic analysis of regulatory mechanisms controlling these degradative pathways is required before it is possible to suggest that positive control might be a common mode of regulation of degradative pathways in *Pseudomonas* spp.

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