p-Cymene Pathway in *Pseudomonas putida*: Selective Enrichment of Defective Mutants by Using Halogenated Substrate Analogs

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Several classes of mutants of *Pseudomonas putida* (JT810) defective in the utilization of *p*-cymene as sole carbon source have been isolated. Selective enrichment of the mutants and for strains putatively cured of a degradative plasmid was achieved by incubation of cells in minimal growth media containing *p*-cymene (or *p*-cumate) and various halogenated analogs of the growth substrates or pathway intermediates. Analogs which led to successful enrichments included: *p*-chlorotoluene, *p*-bromotoluene, α -chloro-*p*-xylene, and *p*-iodobenzoate. A mutant strain, PpJT811, constitutive for the *p*-cymene pathway gave significantly greater enrichments of defective mutants than the wild-type parent PpJT810 after incubation with the halogenated analogs. It is suggested that the defective mutants are enriched because of the genetic alterations they possess, which confer immunity to a lethal synthesis performed by transformation of the analogs in clones possessing an intact *p*-cymene pathway. A nomenclature for the genetic organization of *p*-cymene pathway is described.

Although a large number of degradative pathways have been studied in the nutritionally diverse pseudomonads, there are relatively few reports of the isolation of mutants defective in these dissimilatory pathways. In part, this is probably due to the tedious and medium-consuming manipulations that are frequently employed to obtain them. The development of more expedient mutant isolation procedures may help overcome the lack of strains of pseudomonads defective in catabolic pathways.

The enrichment of mutants defective in a catabolic pathway can be facilitated by the use of a toxic compound or an environment which is lethal for the parent but not the required mutant. Thus antibiotics such as the penicillins and cycloserine have been used for the enrichment of mutants defective in several catabolic pathways in *Pseudomonas putida* (4, 5, 23).

Alternatively, mutants may be enriched using a "suicide compound" (13), that is, a compound which is metabolized to a product that interferes with one or more functions essential for survival or growth under a particular set of environmental conditions. The accumulation of normal metabolites to toxic levels has been achieved in mutant strains unable to metabolize the accumulated intermediate, and this method has been used widely for the isolation of mutants defective in carbohydrate catabolism (12, 14, 15, 17, 20-22, 25). Thus Englesberg et al. (12) isolated a mu-

tant of *Escherichia coli* deficient in L-ribulokinase activity from a parent strain deficient in L-ribulose 5-phosphate 4-epimerase. The epimerase-minus strain but not the wild type was extremely sensitive to L-arabinose, probably due to the accumulation of L-ribulose 5-phosphate in toxic concentrations. Similar mutant strains of *E. coli* have been used for the isolation of mutants defective in the *gal* and *lac* operons, where UDP-galactose epimerase-less (*galE*) strains were used (15, 20, 21, 25), and relief from the intracellular accumulation of UDP-galactose was achieved by mutations inactivating genes, coding for the preceding enzymes responsible for UDP-galactose formation.

The enzymic transformation of a substrate analog to a toxic product was described as a "lethal synthesis" by Peters (24) when he reported that the toxicity of fluoroacetate was due to its conversion to fluorocitrate, a potent inhibitor of aconitase and thus respiration in most strict aerobes. This system has been employed for the enrichment of mutants of *Aspergillus nidulans* unable to grow at the expense of acetate (3) and for the isolation of mutants with altered structural and regulatory genes of aliphatic amidase expression in *A. nidulans* (1, 2, 16) and *Pseudomonas aeruginosa* (7). Extension of these methods by using halogenated analogs seems not to have been generally realized.

The early enzymes of *p*-cymene catabolism from *P. putida* strains JT101 (9, 10) and JT810 (G. J. Wigmore, A. J. Smith, and D. W. Ribbons, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977,

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K188, p. 217) exhibit relaxed substrate and inducer specificity, and they can catalyze the transformation of several halogenated analogs of the pathway intermediates. It seemed that at least some of the halogenated compounds may be substrates for a lethal synthesis and that the transformation(s) may proceed via relatively innocuous intermediates. If this were so, it should be possible to enrich for mutants with defects in the structural and regulatory genes of the early enzymes of the *p*-cymene pathway. *p*-Iodobenzoate was chosen to test the proposed mutant enrichment because this compound was a substrate and inducer for *p*-cumate 2,3-dioxygenase (reaction 4, Fig. 1) of *P. putida* JT101 (9) and JT810 (G. J. Wigmore and D. W. Ribbons, unpublished data). In this communication we report that *p*-iodobenzoate and some other halogenated analogs of *p*-cymene and its catabolites have permitted significant enrichment of mutants defective in early enzymes of the *p*-cymene pathway. Presumably these lesions confer immunity to the presence of these halogenated compounds by preventing a lethal synthesis. The experimental protocol we used, unlike those in the references we have cited, requires that the wild type be killed by an analog intermediate that is formed, but that the mutants survive the treatment and are rescued.

MATERIALS AND METHODS

Bacteria. Strain JT810 was isolated from raw sewage by elective culture with *p*-ethylbenzoate (IV, R = ethyl, Fig. 1) as the sole carbon source. Using the criteria of Stanier et al. (26), it was identified as a *P. putida* of biotype A. This strain also grew with either *p*-cumate (IV, R = isopropyl), *p*-ethyltoluene (I, R = ethyl), or *p*-cymene (I, R = isopropyl) as the sole carbon source. Since each of these growth substrates induced all enzymes of the *p*-cymene pathway as described by DeFrank and Ribbons (9, 10), it was concluded that strain JT810 utilized these compounds according to the scheme in Fig. 1 (Wigmore et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, K188, p. 217). The genes encoding these enzymes have been shown to reside on transmissible plasmids in a number of *p*-cymene utilizers including *P. putida* JT810 (B. N. Anderson, G. J. Wigmore, and D. W. Ribbons, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, O12, p. 199).

Strain JT811 is a spontaneous mutant of strain JT810 which was selected for its ability to grow at the expense of *p*-phenylbenzoate. The 10 enzymes of the *p*-cymene pathway are produced constitutively in strain JT811 (Wigmore et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, K188, p. 217). These and other strains are listed in Table 1.

Maintenance and growth of bacteria. Bacteria were maintained on nutrient agar and grown in R medium, which was prepared as follows: 67 ml of phosphate buffer (136 g of KH_2PO_4 + 25 g of NaOH per liter, pH 6.8), 10 ml of 12% $(\text{NH}_4)_2\text{SO}_4$, and water so that the final volume of complete medium was 1

FIG. 1. Pathway for the metabolism of *p*-substituted toluenes and benzoates with "2,3-dihydroxybenzoates" as ring cleavage substrates (adapted from reference 10). Enzymes (arrows): (1) *p*-cymene hydroxylase; (2) *p*-isopropylbenzyl alcohol dehydrogenase; (3) *p*-isopropylbenzaldehyde dehydrogenase; (4) *p*-cumate 2,3-dioxygenase; (5) dihydrodiol dehydrogenase; (6) 2,3-dihydroxy-*p*-cumate 3,4-dioxygenase; (7) ring cleavage product decarboxylase; (8) hydrolase; (9) hydratase; and (10) aldolase. Symbols I to X refer to the chemical intermediates in the degradative sequence and are explained in the text as the need arises. XI, Pyruvate; XII, acetaldehyde. Letters A, B, C through N refer to the proposed cistrons which encode the enzymes of the degradative sequence. Again, this is explained in the text.

liter; after sterilization and cooling, 7 ml of sterile R salts (800 ml of 10% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200 ml of 1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 4 ml of concentrated HCl) and sterile carbon source were added. All nonvolatile carbon sources were used at a final concentration of 2.5 mM, except glucose, which was used at 10 mM. Volatile carbon sources were introduced as vapors, and when more than one volatile compound was provided, each was present in a separate reservoir. Solid media were prepared by the addition of 1.5% Ionagar no. 2 (Oxoid, Colab Laboratories, Chicago Heights, Ill.). Cultures were incubated at 30 to 33°C.

Mutagenesis and replica-plating. Ethyl methane sulfonate was used for mutagenesis as described previously (4), and a 5% inoculum of the washed mutagenized culture was expressed overnight in either R-glucose or R-*p*-cumate medium before selective pressure was applied. Cultures to be tested for the presence of possible mutants were diluted to obtain isolated colonies. Single colonies were patched on a suitable solid medium by using toothpicks and replicated by using velvet. The presumptive mutants were purified by streaking for single colonies before characterization.

Accumulation of products in culture supernatants. A loopful of an overnight nutrient broth culture of the strain to be tested was inoculated into 10 ml of R-glucose medium in the presence of either *p*-cymene or *p*-cumate and incubated for 3 days. After 2 and 3 days of incubation, portions of the culture supernatants were diluted 50-fold for spectrophotometric determinations between 230 and 350 nm. For preliminary characterization of products formed, the following λ_{max} values were used: *p*-cumate, 235 nm; "dihydrodiol," 293 nm; and 2,3-dihydroxy-*p*-cumate, 245 and

305 nm. The identity of products formed was confirmed after isolation of the products from 2-liter cultures and spectral and degradative analysis (Wigmore, DeFrank, and Ribbons, manuscript in preparation).

Cell extracts. Extracts were prepared as previously described (9), except that the high-speed centrifugation was omitted.

Enzyme assays. The activities of *p*-cymene hydroxylase (reaction 1, Fig. 1), *p*-cumate 2,3-dioxygenase (reaction 4, Fig. 1), and 2,3-dihydroxy-*p*-cumate 3,4-dioxygenase (reaction 6, Fig. 1) were determined polarographically using washed whole-cell suspensions as described previously (9). Occasionally the same method was used to assay 2,3-dihydroxy-*p*-cumate 3,4-dioxygenase in cell-free extracts. The substrates were dissolved in buffer, except for *p*-cymene, which was dissolved in isopropanol.

p-Isopropylbenzyl alcohol dehydrogenase (reaction 2, Fig. 1) and *p*-isopropylbenzaldehyde dehydrogenase (reaction 3, Fig. 1) were assayed by spectrophotometric measurement of NADH formation at 340 nm in reaction mixtures containing 2.85 ml of 50 mM glycine-NaOH (pH 9.5), 50 μl of cell extract, 1.25 μmol of NAD, and 1.25 μmol of substrate dissolved in isopropanol. The dihydrodiol dehydrogenase (reaction 5, Fig. 1) was assayed as described previously (9), except that the buffer used was 50 mM glycine-NaOH (pH 9.5). "Ring-cleavage product decarboxylase" (reaction 7, Fig. 1) was assayed spectrophotometrically by following the disappearance at 345 nm of the substrate formed in the following reaction mixture: 50 mM KH_2PO_4 -NaOH (pH 7.5, 2.8 ml), 25 mM 2,3-dihydroxy-*p*-cumate (10 μl), and cell-free extract (50 μl) prepared from *P. putida* JT104 grown in the presence of *p*-cumate. The hydrolase (reaction 8, Fig. 1) was assayed

TABLE 1. Assignment of strain collection numbers to the *P. putida* mutants described in this paper

Strain collection no.	Isolation no. ^a	Immediate parent	Phenotype ^b	Genotype ^c
JT810	—	—	Cym ⁺	<i>cym</i> ⁺
JT811	—	JT810	Cym ⁺ Ppb ⁺	<i>cymR1</i>
JT831	1	JT810	Cym ⁻	<i>cymF2</i>
JT832	8	JT811	Cym ⁻ Ppb ⁻	<i>cymR1 cymI3</i>
JT833	7	JT811	Cym ⁻ Ppb ⁻	<i>cymR1 cymJ4</i>
JT834	W-40	JT811	Cym ⁻ Ppb ⁻	<i>cymR1 cymJ5</i>
JT835	W-80	JT811	Cym ⁻ Ppb ⁻	<i>cymR1 cymI6</i>
JT836	W-3	JT811	Cym ⁻ Ppb ⁺ Cum ⁺	<i>cymR1 cym-7</i>
JT837	W-6	JT811	Cym ⁻ Ppb ⁺ Cum ⁺	<i>cymR1 cym-8</i>
JT838	W-17	JT811	Cym ⁻ Ppb ⁺ Cum ⁺	<i>cymR1 cym-9</i>
JT839	W-19	JT811	Cym ⁻ Ppb ⁺ Cum ⁺	<i>cymR1 cym-10</i>
JT840	W-20	JT811	Cym ⁻ Ppb ⁺ Cum ⁺	<i>cymR1 cym-11</i>
JT841	X-18	JT811	Cym ⁻ Ppb ⁺ Cum ⁺	<i>cymR1 cym-12</i>
JT842	D-7	JT810	Cym ⁻ Cum ⁺	<i>cym-13</i>
JT843	D-73	JT810	Cym ⁻ Cum ⁺	<i>cym-14</i>
JT844	I-3	JT810	Cym ⁻	<i>cymI15</i>
JT104	PL-RF1 ^d	JT101 ^e	Cym ⁻	<i>cymK71</i>
JT006	O1 ^f			

^a As used in the text.

^b Phenotypes. Cym, Ppb, and Cum refer to the ability to grow with *p*-cymene, *p*-phenylbenzoate, and *p*-cumate, respectively, as the sole carbon source.

^c The assignment of the genetic designations for the various classes of mutants is discussed in the text.

^d Defective in decarboxylase, reaction 7, Fig. 1.

^e *P. putida* PL-W (10).

^f *P. putida* O1 (6).

spectrophotometrically by following the disappearance at 393 nm of the substrate formed in the following reaction mixture: 50 mM KH_2PO_4 -NaOH (pH 7.5, 2.8 ml), 25 mM 3-isopropylcatechol (15 μl), and extracts of orcinol-grown *P. putida* (JT006; reference 6). At pH 7.5 the molar extinction coefficients for the decarboxylase and hydrolase substrates were taken to be 3.03×10^4 and 1.00×10^4 , respectively (Lampel, unpublished data). The hydratase (reaction 9, Fig. 1) was assayed as described by Collinsworth et al. (8). The aldolase (reaction 10, Fig. 1) was not assayed due to difficulties encountered in obtaining reproducible and significant specific activities; specific activities reported for this enzyme have been consistently low.

Other methods. Spectrophotometric measurements were made as previously described (9). All commercially available chemicals were used without further purification, except *p*-cumate, which was always recrystallized from water.

RESULTS

Enrichment of mutants in the presence of *p*-iodobenzoate. Loopfuls of overnight nutrient broth cultures of unmutagenized strains JT810 and JT811 were used to inoculate separate 10-ml quantities of R medium containing *p*-cumate plus *p*-iodobenzoate as the sole carbon sources. The cultures were incubated without shaking for 2 days and were then plated for single colonies on R-glucose medium. Seventy-six single colonies of each were patched onto R-glucose medium, and after overnight incubation, they were replicated onto (i) R-*p*-cumate and (ii) R-glucose media.

Four of the 76 patches derived from JT810 and 38 of the 76 patches derived from JT811 failed to grow at the expense of *p*-cumate. This growth response was confirmed after plating for single colonies. The four JT810 isolates (no. 1 to 4) and eight of the JT811 isolates (no. 5 to 12) which failed to grow on *p*-cumate were selected for further study. These presumptive *p*-cumate-defective mutants were otherwise identical to the parent strain as shown by various nutritional tests and their colonial appearance on and the pigments excreted into the media A and B of King et al. (18). The selected *p*-cumate-negative strains were tested for their ability to oxidize *p*-cumate, 2,3-dihydroxy-*p*-cumate, or both (Table 2). These observations, together with the spectra of products accumulated after growth of each of the selected putative mutants in R-glucose medium in the presence of either *p*-cumate or *p*-cymene, suggested the tentative identification of lesions presented in Table 3. The identity of the UV-absorbing compounds that were accumulated was determined after the isolation of the material from 2-liter cultures. The compound that accumulated from *p*-cymene by isolate no. 1 and which absorbed maximally at 235 nm was

TABLE 2. Oxygen consumption of selected putative mutants

Strain	Respiratory rate ^a					
	Glucose ^b			Glucose + <i>p</i> -cumate ^b		
	None ^c	<i>p</i> -Cu-mate ^c	DCA ^c	None	<i>p</i> -cumate	DCA
JT810	27	27	27	29	77	70
No. 1	25	25	25	21	21	66
No. 2	23	23	23	23	23	23
JT811	24	70	63	27	108	72
No. 5	25	25	25	26	26	26
No. 7	20	30 ^d	20	23	45 ^d	23
No. 8	25	32 ^d	83	27	36 ^d	85

^a Expressed as nanomoles of O_2 consumed per minute per milligram (dry weight) of cells. Endogenous respiration rates have not been subtracted.

^b Growth substrate(s).

^c Assay substrate. DCA, 2,3-Dihydroxy-*p*-cumate.

^d The partial stimulation of oxygen consumption is presumably due to a block in a subsequent enzyme and hence prevents "amplification" of the oxygen consumption.

shown to be *p*-cumate. Isolates no. 7 and 8 accumulated 2,3-dihydroxy-*p*-cumate (V, R = isopropyl, Fig. 1; λ_{max} , 245 and 305 nm) and the "dihydrodiol" (VI, R = isopropyl, Fig. 1; λ_{max} , 295 nm), respectively. 3-Hydroxy-*p*-cumate was isolated from acidified, clarified culture supernatants of isolate no. 8 (9). Details of the isolation, purification, and identification of these compounds will be presented elsewhere (Wigmore et al., in preparation). Seven survivors of a second experiment, in which a nonmutagenized culture of strain JT810 was incubated for 3 days in R media containing *p*-cumate (2.5 mM) and *p*-iodobenzoate (2.5 mM), were tested. One isolate, designated I-3 (Table 1), presumably had a defective dihydrodiol dehydrogenase, as this strain accumulated a product of λ_{max} 293 nm from *p*-cumate.

The identity of each of the assigned lesions presented in Table 3 was confirmed by enzyme assay. Each of the mutant strains was shown to have wild-type levels of all enzymes of the *p*-cymene pathway except that of the assigned defect(s). In addition, the two isolates obtained from JT811, which were selected for further study, namely, no. 7 and 8, retained their ability to constitutively express the other genes of the *p*-cymene pathway (data not shown).

Halogenated analogs of *p*-cumate as possible enrichment agents. Additional compounds were tested for their ability to selectively enrich for strains unable to grow at the expense of *p*-cumate. A loopful of mutagenized cultures of *P. putida* JT810 and JT811 which had been allowed to express in R-glucose media was in-

TABLE 3. λ_{max} values of culture supernatants after potential mutants of strains JT810 and JT811 were exposed to either *p*-cymene or *p*-cumate

Strain	λ_{max} with aromatic compound provided:		Identification of lesions in mutant strains ^a
	<i>p</i> -Cymene	<i>p</i> -Cumate	
JT810	NP ^b	NP	—
JT811	NP	NP	—
No. 1	235 nm	235 nm ^c	<i>p</i> -Cumate 2,3-dioxygenase defective
Nos. 2, 3, 4, 5, 6, 9, 12	NP	235 nm ^c	Cured ^d
No. 7	245 and 305 nm	245 and 305 nm	2,3-Dihydroxy- <i>p</i> -cumate 3,4-dioxygenase defective
Nos. 8, 10, 11	293 nm	293 nm	Dihydrodiol dehydrogenase defective

^a Refer to Fig. 1.^b NP, No UV-absorbing product detected.^c Spectra unchanged during incubation.^d These strains do not revert, and one designated JT820, unlike its parent JT811, does not yield plasmid DNA bands after lysates are banded in cesium chloride-ethidium bromide gradients (Anderson et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, O12, p. 199).

oculated into separate 10-ml amounts of R media containing 2.5 mM *p*-cumate and equimolar quantities of each of the halogenated analogs. The cultures were incubated without shaking for 7 days. At 3 and 7 days, the enrichment cultures were examined for growth, as *p*-cumate-negative strains were only expected to be enriched in those cultures that had not grown. After 3 days of incubation, those cultures that failed to exhibit significant growth were diluted and plated for single colonies. In each of the cultures diluted for single colonies there were a significant number of survivors (Table 4). The colonies were tested for their ability to grow at the expense of *p*-cumate as described above. Of the compounds tested, only *p*-iodobenzoate gave significant enrichment of *p*-cumate-defective strains (Table 5). These strains were not characterized further. Halogenated analogs which failed to inhibit growth with *p*-cumate included *p*-fluorobenzoate, α -bromo-*p*-toluate, 3-chloro-4-toluate, 3,4-dichlorobenzoate, and 4-bromo-3-methylbenzoate.

Volatile halogenated analogs as enrichment agents. Analogs of the first three compounds (I, II, and III, Fig. 1) of the *p*-cymene pathway were also tested using the same mutagenized cultures as were used in the preceding description. These compounds were provided as vapors and were contained in culture tubes (6 by 50 mm) as free-standing reservoirs in 50-ml Erlenmeyer flasks. Survivors were tested from incubation mixtures that failed to exhibit significant growth after 3 days of incubation (Table 6). They were patched onto R-glucose medium, replicated onto R agar, and exposed to *p*-cymene vapor. All of the survivors from strain JT810 were able to grow at the expense of *p*-cymene (Table 7). However, the presence of three com-

pounds, namely, α -chloro-*p*-xylene (I, R = chloromethyl, Fig. 1), *p*-chlorotoluene (I, R = Cl, Fig. 1), and *p*-bromotoluene (I, R = Br, Fig. 1), provided an enrichment for mutants of JT811 unable to grow at the expense of *p*-cymene (Table 7). The putative mutants unable to grow at the expense of *p*-cymene were tested for their ability to grow at the expense of *p*-cumate. Six isolates were able to grow with *p*-cumate but not *p*-cymene (Table 8). Strains unable to grow with either *p*-cymene or *p*-cumate were inoculated into R-glucose plus *p*-cumate medium so that mutants could be tentatively identified. After 2 days, the culture supernatants were examined spectroscopically between 220 and 350 nm. Mutants defective in dihydrodiol dehydrogenase (reaction 5, Fig. 1) and 2,3-dihydroxy-*p*-cumate 3,4-dioxygenase (reaction 6, Fig. 1) activity were present. It was not possible to distinguish mutants defective in *p*-cumate 2,3-dioxygenase (reaction 4, Fig. 1) from regulatory mutants and cured strains in this experiment, because *p*-cumate will remain unmetabolized by both classes of defective strains.

The failure to isolate mutants of strain JT810 blocked between *p*-cymene and *p*-cumate prompted the design of alternative strategies to improve the selection. One modification was to express the mutagenized culture in *p*-cumate instead of glucose medium. This would eliminate cured strains among the survivors and induce the *p*-cymene enzymes before selection. Preinduction of the pathway with *p*-cumate may favor enrichment since it had been consistently observed that the constitutive strain JT811 yielded more mutants defective in *p*-cymene and *p*-cumate metabolism than strain JT810. Another possible modification for the elimination of cured strains was to isolate the survivors on R-

TABLE 4. Growth response and presence of survivors when mutagenized cultures of strains JT810 and JT811 were incubated in the presence of *p*-cumate plus a halogenated analog of *p*-cumate

Analog	JT810			JT811		
	Growth		Survivors after 3 days	Growth		Survivors after 3 days
	3 days	7 days		3 days	7 days	
<i>p</i> -Chlorobenzoate	—	2+	Yes	+	2+	Yes
<i>p</i> -Bromobenzoate	—	—	Yes	±	2+	Yes
<i>p</i> -Iodobenzoate	—	—	Yes	—	—	Yes
α,α,α -Trifluoro- <i>p</i> -toluate	—	2+	Yes	—	2+	Yes

TABLE 5. Number of survivors unable to grow at the expense of *p*-cumate after 3 days of incubation in the presence of halogenated analogs of *p*-cumate

Analog	JT810	JT811
<i>p</i> -Chlorobenzoate	0 (76) ^a	ND ^b
<i>p</i> -Bromobenzoate	0 (76)	0 (76)
<i>p</i> -Iodobenzoate	26 (76)	69 (76)
α,α,α -Trifluoro- <i>p</i> -toluate	0 (100)	0 (76)

^a Numbers in parentheses are the number of survivors tested.

^b ND, Not determined.

p-cumate rather than R-glucose medium. Additionally, it was possible that D-cycloserine may be synergistic with the substrate analogs in killing wild-type cells. Carhart and Hegeman (5) described a selection using D-cycloserine in which the antibiotic was added, at intervals, to the selection medium. In several experiments D-cycloserine was added (to give a final concentration of each addition of 5 μ g/ml) at 8-h intervals (for a total of 10 additions) with α -chloro-*p*-xylene as the substrate analog, for possible synergistic effects. After several attempts using the proposed modifications and changing such parameters as incubation time, aeration, and inoculum size, no mutants of strain JT810 were isolated that could grow with *p*-cumate but not *p*-cymene as the sole carbon source. In these experiments α -chloro-*p*-xylene was used as the possible selective compound.

Since the success of selections employing recycling has been well demonstrated (19, 23) it was decided to use a protocol which used recycling so that enrichment for mutants of *P. putida* JT810 blocked between *p*-cymene and *p*-cumate might be achieved.

Strain JT810 was exposed to ethyl methane sulfonate and allowed to express overnight in R-*p*-cumate medium. This culture was used to inoculate R medium and was exposed to α -chloro-*p*-xylene and *p*-cymene vapors for 3 days. A sample (0.2 ml) from the enrichment medium was transferred to 10 ml of R-*p*-cumate medium, and the culture was incubated overnight. The survivors from a second round of enrichment in the presence of α -chloro-*p*-xylene-*p*-cymene

were isolated on R-*p*-cumate agar and tested for their ability to grow with *p*-cymene as the sole carbon source. Of 304 colonies tested, 294 were unable to grow with *p*-cymene but grew with *p*-cumate. Because of the recycling it would be expected that many of the isolates would be siblings.

Preliminary characterization of mutants isolated using the volatile halogenated analogs. The six mutants derived from strain JT811 able to grow with *p*-cumate but not *p*-cymene retained their ability to constitutively express all enzymes of the pathway that were present in extracts prepared from cells grown in the presence of *p*-cumate. Four of the mutants, W-3, -17, -19, and -20 (Table 8), retained wild-type levels of the alcohol and aldehyde dehydrogenases (reactions 2 and 3, Fig. 1). Since the oxygen consumption of whole cells was not stimulated by the addition of *p*-cymene, these four mutants are apparently defective in *p*-cymene hydroxylase (reaction 1, Fig. 1).

It has not been possible to detect either alcohol or aldehyde dehydrogenase activity in extracts prepared from cells of W-6 and X-18 (Table 8). The pleiotropy of the lesion in these two strains may also involve the *p*-cymene hydroxylase since there was no detectable stimulation of oxygen consumption by whole cells on the addition of *p*-cymene. The nature of the lesion that has resulted in this pleiotropic effect is not understood, but it may represent another example of the loss of a block of enzymes used for the oxidation of a methyl group to carboxyl (Kunz and Chapman, personal communication).

Five of the clones derived from strain JT810, which were isolated after repeated exposure to α -chloro-*p*-xylene, were tested for the presence of alcohol, aldehyde, and dihydrodiol dehydrogenases (reactions 2, 3, and 5, Fig. 1) in cells grown in the presence of *p*-cumate. The three dehydrogenases were present at wild-type levels in all isolates. Five of these isolates were then examined for the presence of these enzymic activities after growth in R-glucose medium, with exposure to *p*-cymene vapor throughout growth. Four of these mutants, including an isolate designated D-7, had markedly reduced (0 to 10%)

TABLE 6. Growth response and presence of survivors when mutagenized cultures of strains JT810 and JT811 were incubated in the presence of *p*-cymene plus a volatile analog of either *p*-cymene or its catabolites

Analog	JT810			JT811		
	Growth		Survivors after 3 days	Growth		Survivors after 3 days
	3 days	7 days		3 days	7 days	
α -Chloro- <i>p</i> -xylene	—	—	Yes	—	—	Yes
<i>p</i> -Chlorotoluene	—	—	Yes	—	—	Yes
<i>p</i> -Bromotoluene	—	—	Yes	—	—	Yes
4-Fluorobenzyl alcohol	—	2+	Yes	2+	ND ^a	ND
4-Chlorobenzyl alcohol	—	—	No	—	—	No
4-Fluorobenzaldehyde	—	—	Yes	—	2+	Yes
4-Chlorobenzaldehyde	—	—	No	—	—	No
4-Bromobenzaldehyde	—	—	No	—	—	No
4-Chloro-3-nitrotoluene	2+	ND	ND	2+	ND	ND

^a ND, Not determined.

TABLE 7. Number of survivors unable to grow at the expense of *p*-cymene after incubation in the presence of the volatile halogenated analogs

Analog	JT810	JT811
α -Chloro- <i>p</i> -xylene	0 (152) ^a	18 (125)
<i>p</i> -Chlorotoluene	0 (76)	39 (125)
<i>p</i> -Bromotoluene	0 (76)	9 (76)
4-Fluorobenzyl alcohol	0 (152)	ND ^b
4-Fluorobenzaldehyde	0 (152)	0 (76)

^a Numbers in parentheses are the number of survivors tested.

^b ND, Not determined.

TABLE 8. Partial characterization of potential mutants of strain JT811 unable to grow at the expense of *p*-cymene

Analog used for enrichment	No. of potential mutants tested	No. able to grow with <i>p</i> -cymate
α -Chloro- <i>p</i> -xylene	23	5 ^{a, b, c}
<i>p</i> -Chlorotoluene	49	1 ^{a, b, d}
<i>p</i> -Bromotoluene	7	0 ^b

^a Among potential mutants unable to grow with *p*-cymate, 2,3-dihydroxy-*p*-cymate 3,4-dioxygenase mutants were isolated.

^b Among potential mutants unable to grow with *p*-cymate, "dihydrodiol" dehydrogenase mutants were isolated.

^c Strains were designated W-3, W-6, W-17, W-19, and W-20.

^d Strain was designated X-18.

levels of all three dehydrogenases. The activity of other enzymes of the *p*-cymene pathway, namely, hydrolase (reaction 8, Fig. 1) and hydratase (reaction 9, Fig. 1), were concomitantly lowered. These strains may have a mutation which reduces the ability of the regulatory protein to functionally recognize *p*-cymene as an

allosteric effector. The fifth isolate, which had been designated D-73, retained wild-type levels of the three dehydrogenases after growth in the presence of *p*-cymene. It has not been possible to detect an active *p*-cymene hydroxylase (reaction 1, Fig. 1) in washed whole cells in this strain. The biochemical details of these mutants will be presented elsewhere.

DISCUSSION

Mutations in structural or regulatory genes causing defects in acetate and acetamide utilization have been assumed to be the basis of the enrichment of strains resistant to a lethal synthesis performed after cells were exposed to fluorinated substrate analogs (1-3, 7, 16). An alternate mode of resistance to a lethal synthesis has been described in *E. coli* (13). Mutants that are resistant to methylglyoxal, the toxic substrate which accumulates during unregulated glycerol metabolism, are resistant as a consequence of an enhanced ability to metabolize the lethal product.

It has been assumed that the biochemical basis for the enrichment procedure we have described is the acquisition of resistance to a lethal synthesis, this being conferred by the inability to express one or more enzymes of the *p*-cymene pathway that participate in the transformation of a halogenated substrate analog to a lethal metabolite. We have no direct evidence to support this contention. *p*-Halo-substituted toluenes and benzoates have been assumed to exert their selective influence as a consequence of the formation of "6-carboxy-3-halo-catechols" (VI, Fig. 1), the substrate analogs of ring cleavage, or acyl halides by dioxygenative ring cleavage (VII, Fig. 1) if their metabolism proceeds this far. α -Chloro-*p*-xylene (I, R = chloromethyl, Fig. 1) may exert its selective influence by for-

mation of a reactive and lethal chloromethylketone (VII, R = chloromethyl, Fig. 1) as a product of ring cleavage (reaction 6, Fig. 1). Consistent with these proposals is the fact that mutants have not been isolated which are defective in enzymes later in the reaction sequence than 2,3-dihydroxy-*p*-cumate 3,4-dioxygenase (reaction 6, Fig. 1).

We have made no attempt to define the conditions for optimum enrichment, and obviously these will vary from system to system. Among the parameters that must be considered are the relative and absolute concentrations of substrate/inducer and analog, incubation times, aeration, preinduction, and the use of possible synergistic reagents. One important variable appears to be the analog concentration, since in at least one pathway, that for 4-methyl-*o*-phthalate utilization in *Pseudomonas fluorescens* (B. N. Anderson, personal communication), high absolute or relative concentrations of the analog select against mutants among the survivors. Although the physiological basis of this is not known, the higher concentrations of analog may inhibit a lethal synthesis by inhibition of either an enzyme of the reaction sequence or the expression of the genes determining the sequence. In this respect we have found that strains that constitutively produce the *p*-cymene enzymes were more amenable to the isolation of structural gene mutations. The amidase-defective mutants of both *A. nidulans* and *P. aeruginosa*, which were isolated as fluoroacetamide-resistant mutants, were derived mainly from strains which produced the amidase constitutively (1, 7).

A nomenclature for the mutant strains. Since the mutant strains described in this manuscript will be the subject of subsequent communications, it is opportune to introduce a genetic nomenclature in accordance with the proposals of Demerec et al. (11). A number of points need to be considered, and assumptions must be made.

Although there are 10 enzymic steps in the *p*-cymene pathway (9, 10), there are more than 10 loci determining the expression of the entire degradative sequence. Two of the enzymes, namely, *p*-cymene hydroxylase (reaction 1, Fig. 1) and *p*-cumate 2,3-dioxygenase (reaction 4, Fig. 1), are likely to be multicomponent enzymes; each enzyme may be encoded by as many as three cistrons, as described for similar mono- and dihydroxylases. In anticipation of this, three loci, namely, *cymA*, *cymB*, and *cymC*, have been assigned to define the genetic determinants specifying *p*-cymene hydroxylase. Similarly, *cymF*, *cymG*, and *cymH* have been assigned to the loci determining *p*-cumate 2,3-dioxygenase. As the

other enzymes of the reaction sequence are likely to be single gene products, and in fact this has been demonstrated for several of the enzymes (K. Lampel, Ph.D. dissertation, University of Miami, Miami, Fla., 1979), a single locus for each of the remaining enzymes seems adequate. It is possible that more than one isofunctional locus exists for some enzymes of the pathway.

In addition, one locus determining the control of the pathway can be defined. We have assigned *cymR* to be one such locus—it is arbitrarily defined as that control locus which in strain JT811 was altered to allow constitutive synthesis of all the *p*-cymene enzymes. This assignment is not meant to have any specific connotations; it could equally be the site encoding a regulatory protein or its site of action. The assignment of genotypes for the mutant strains we have isolated and retained is summarized in Table 1.

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