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Possible Regulatory Role for Nonaromatic Carbon Sources in Styrene Degradation by *Pseudomonas putida* CA-3

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Styrene metabolism in styrene-degrading *Pseudomonas putida* CA-3 cells has been shown to proceed via styrene oxide, phenylacetaldehyde, and phenylacetic acid. The initial step in styrene degradation by strain CA-3 is oxygen-dependent epoxidation of styrene to styrene oxide, which is subsequently isomerized to phenylacetaldehyde. Phenylacetaldehyde is then oxidized to phenylacetic acid. Styrene, styrene oxide, and phenylacetaldehyde induce the enzymes involved in the degradation of styrene to phenylacetic acid by *P. putida* CA-3. Phenylacetic acid-induced cells do not oxidize styrene or styrene oxide. Thus, styrene degradation by *P. putida* CA-3 can be subdivided further into an upper pathway which consists of styrene, styrene oxide, and phenylacetaldehyde and a lower pathway which begins with phenylacetic acid. Studies of the repression of styrene degradation by *P. putida* CA-3 show that glucose has no effect on the activity of styrene-degrading enzymes. However, both glutamate and citrate repress styrene degradation and phenylacetic acid degradation, showing a common control mechanism on upper pathway and lower pathway intermediates.

The use of synthetic aromatic hydrocarbons has, in many cases, resulted in their release into the environment. Improper disposal and inadequate control of toxic materials have led to widespread contamination of soils, groundwater, and surface water bodies. Styrene is used in the chemical industry both as a starting material for synthetic polymers and as a solvent in the polymer processing industry and consequently is present in many industrial effluents. Airborne gaseous emissions of styrene are of particular importance because of the malodorous properties of the compound and its potential toxic and carcinogenic properties. However, styrene can also occur naturally in the environment as a result of the decay of organic materials, i.e., the decarboxylation of cinnamic acid (15).

Previous studies of styrene catabolism have shown the formation of 2-phenylethanol via styrene oxide by microorganisms grown on styrene (16). Two independent studies of *Pseudomonas* strains have previously proposed that styrene degradation proceeds via phenylacetic acid (2, 17). A recent study by Warhurst et al. of *Rhodococcus* strain NCIMB 13259 has shown that styrene degradation proceeds via styrene *cis*-glycol and 3-vinylcatechol, which is then either oxidized to the dead-end product 2-vinyl-*cis*,*cis*-muconate because of *ortho* cleavage or completely metabolized via a *meta* cleavage pathway (18). This differs significantly from the results of other studies of styrene degradation. Catabolite repression in many *Pseudomonas* species has been well documented for both nonaromatic (11, 12) and aromatic carbon sources (9, 19). The ability of a carbon source to repress metabolism of an aromatic carbon source is beneficial to the organism but may ultimately affect its potential use in bioremediation strategies. Here we report nonaromatic carbon source-dependent repression of styrene degradation by *Pseudomonas putida* CA-3. In addition, differences in the induction of styrene degradation by aromatic carbon sources suggest a division in this degradative pathway.

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MATERIALS AND METHODS

Growth conditions. *P. putida* CA-3 cells used in this study were isolated from a bioreactor following enrichment on styrene. Cultures were grown in 1-liter Erlenmeyer flasks in 100 ml of minimal salts (MS) medium. MS medium contained 7.0 g of K_2HPO_4, 3.0 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, and 2 ml of 10% (wt/vol) $MgSO_4 \cdot 7H_2O$ per liter of demineralized water. All carbon sources were added at optimal concentrations for growth. Glucose and acetate were added at concentrations of 0.05%. Glutamate and citrate were added at concentrations of 0.07 and 0.1%, respectively. Sodium succinate was added at concentrations of 0.05% for induction studies and 0.1% for studies of specific enzyme activities. The aromatic carbon source phenylacetic acid was added directly to the medium before autoclaving at a concentration of 0.1% (wt/vol). 2-Phenylethanol was added directly to the medium after autoclaving at an initial concentration of 0.01% (vol/vol). This concentration was increased gradually to 0.1% (vol/vol) after growth on 2-phenylethanol was seen. Fifty microliters of liquid styrene or styrene oxide or 30 μl of phenylacetaldehyde was added to a test tube fixed in a central position at the base of the 1-liter Erlenmeyer flask. Flasks were incubated at 30°C with shaking at 120 rpm. Culture doubling times were determined by monitoring the increase in optical density at 600 nm (OD_{600}) with a Beckman DU640 spectrophotometer. For whole-cell induction studies, inducers were added at time zero. For enzyme activity and whole-cell induction studies, cultures were harvested in mid-log phase (OD_{600}, 0.3 to 0.5), concentrated by centrifugation, washed, and resuspended in 50 mM potassium phosphate (pH 7.0). Cell extracts for enzyme activity studies were prepared by sonication and kept on ice at all times (7). For whole-cell repression experiments, cultures were harvested at various stages of growth and treated in an identical manner to cultures used in induction studies.

Enzyme assays. All assays were performed at 30°C with extracts from freshly harvested cells. Activities were expressed in nanomoles of product formed (NADH or NADPH) or substrate consumed (styrene or O_2) $minute^{-1}$ mg of $protein^{-1}$. One unit was the amount of enzyme that catalyzed the transformation of 1 μmol of substrate per min. Phenylacetaldehyde dehydrogenase (phenazine methosulfate [PMS]-dependent) activities were assayed by determining oxygen consumption rates as previously described (7). Styrene oxide isomerase (SOI), phenylacetaldehyde dehydrogenase, and 2-phenylethanol dehydrogenase ($NAD(P)^+$-dependent) activities were assayed spectrophotometrically as previously described (7). In the SOI assay, the cell extract from 2-phenylethanol-grown *P. putida* LW4 cells (7) was replaced by an extract from 2-phenylethanol-grown strain PE1 cells, spore-forming rods, tentatively identified as a *Bacillus* species. This extract was devoid of SOI activity and contained high levels of NAD^+-dependent phenylacetaldehyde dehydrogenase activity. Fresh styrene oxide stock solutions were prepared by adding 10 μl of styrene oxide to 10 ml of demineralized water. Styrene monooxygenase (SMO) activity was measured by determining the degradation of styrene by gas chromotography as previously described (8).

Oxygen uptake experiments. Oxygen uptake experiments were performed with washed whole cells in a total volume of 3.0 ml of 50 mM potassium phosphate buffer (pH 7.0) at 30°C (8). The endogenous O_2 consumption rate was monitored

TABLE 1. Growth of *P. putida* CA-3 cells on various carbon sources

Carbon source	Doubling time (h)
Styrene	2
Styrene oxide	10
1-Phenylethanol	NG[a]
Phenylacetaldehyde	23
Phenylacetic acid	1

[a] NG, no growth.

for 5 min. Then 0.1 ml of a 10 mM substrate solution was added to the reaction mixture, and the oxygen uptake rate was monitored for at least another 5 min. All substrate solutions were made up fresh to a concentration of 10 mM. Styrene, styrene oxide, and phenylacetaldehyde are only slightly soluble in water and therefore exist as water-saturated solutions. Oxygen-dependent degradation of styrene by washed cell suspensions of styrene-grown cells on styrene was determined as previously described (7).

Analytical methods. Protein concentrations were determined by the method of Lowry et al. (10), with bovine serum albumin as the standard protein solution. Spectrophotometric assays were performed on a Beckman DU 640 series spectrophotometer. Oxygen uptake experiments were carried out with a Rank biological oxygen analyzer.

Chemicals. Styrene; styrene oxide; 1- and 2-phenylethanol; propyl-, butyl-, and ethylbenzene; phenylacetaldehyde; phenylacetic acid; sodium succinate; glucose; citrate; glutamate; PMS; and crystallized bovine serum albumin were supplied by Sigma Chemical Co., St. Louis, Mo.

RESULTS

Growth experiments. *P. putida* CA-3 cells grew on a variety of carbon sources, including styrene, styrene oxide, phenylacetaldehyde, and phenylacetic acid (Table 1). *P. putida* CA-3 cells grew with a short lag phase on styrene and phenylacetic acid and with a longer lag phase on styrene oxide and phenylacetaldehyde. The lowest growth rates were observed when either styrene oxide or phenylacetaldehyde was the sole carbon source (Table 1). This is possibly due to the toxic nature of these compounds (7). *P. putida* CA-3 cells do not grow on propyl-, butyl-, or ethylbenzene; 1-phenylethanol; or benzene. When *P. putida* CA-3 cells were grown in liquid culture with phenylacetic acid, the transient accumulation of a yellow color was observed as cells entered the stationary phase of growth. A pH-dependent shift in the absorbance maxima for the accumulating compound from 361 nm at pH 2 to 413 nm at pH 11 was observed.

Oxygen-dependent styrene degradation. Styrene degradation by *P. putida* CA-3 was oxygen dependent; the removal of oxygen by flushing the reaction vial with nitrogen gas before the addition of styrene resulted in no styrene consumption.

TABLE 2. Specific enzyme activities in crude cell extracts of *P. putida* CA-3 grown on various carbon sources

Enzyme	Sp act of cells grown on[a]:				
	Styrene	Styrene oxide	Phenylacetaldehyde	Phenylacetic acid	Succinate
Phenylacetaldehyde dehydrogenase					
PMS dependent	47	73	72	14	15
NAD^+ dependent	52	68	65	15	10
$NADP^+$ dependent	<5	<5	<5	66	<5
SOI	328	463	440	<5	<5
SMO	11	—[b]	—	—	—

[a] Specific enzyme activity was measured in nanomoles min^{-1} mg of $protein^{-1}$. Data are averages of at least three independent determinations.

[b] —, not determined.

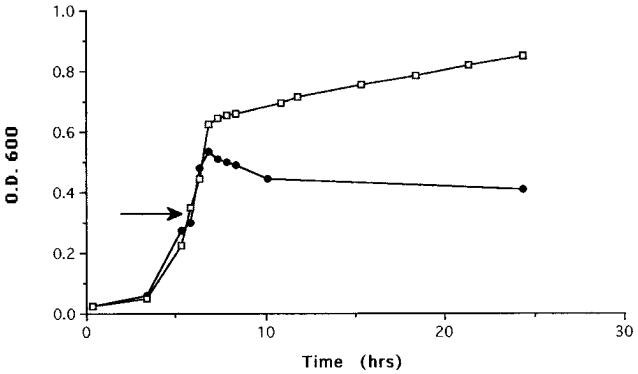

FIG. 1. Growth of *P. putida* CA-3 cells under nonrepressing conditions. Growth of *P. putida* CA-3 cells in MS medium which contained 0.05% glucose (●) or 0.05% glucose and styrene (□). Cells were harvested for substrate utilization experiments at the OD_{600} indicated by the arrow.

The subsequent addition of oxygen to the reaction vial saw a restoration of the oxidation rate of styrene to that of an aerobic control. Respiration studies were conducted by monitoring substrate-dependent oxygen utilization rates of washed cell suspensions. These studies show that styrene, styrene oxide, phenylacetaldehyde, and phenylacetic acid are oxidized to various degrees according to the sole carbon source on which washed cells were initially grown (data not shown).

Enzyme activities. To further characterize the styrene-degradative pathway, specific enzyme activities were studied. In crude cell extracts of *P. putida* CA-3, PMS- and NAD^+-dependent phenylacetaldehyde dehydrogenase activities were detected in cells grown on styrene, styrene oxide, and phenylacetaldehyde (Table 2), with approximately the same relative levels of PMS- and NAD^+-dependent activities. Phenylacetic acid- and succinate-grown cells were found to have low constitutive levels of PMS- and NAD^+-dependent phenylacetaldehyde dehydrogenase activities. High levels of $NADP^+$-dependent phenylacetaldehyde dehydrogenase activity were detected in phenylacetic acid-grown cells but were not detected in extracts of cells grown on all of the other carbon sources tested. PMS-dependent phenylacetaldehyde dehydrogenase activity was not stimulated by NH_4Cl. SOI activity was found to be high in styrene-grown cells as well as styrene oxide- and phenylacetaldehyde-grown cells but was not detected in phenylacetic acid- and succinate-grown cells of strain CA-3. The SMO activity of *P. putida* CA-3 was assayed only in extracts of styrene-grown cells.

TABLE 3. Carbon source-dependent induction of styrene degradation by *P. putida* CA-3

Substrate	Rate of oxygen uptake[a]				
	Styrene	Styrene oxide	Phenylacetaldehyde	Phenylacetic acid	Glucose
Styrene	370	183	92	<5	<5
Styrene oxide	539	984	241	<5	<5
Phenylacetaldehyde	539	470	335	315	<5
Phenylacetic acid	498	336	229	297	<5
Glucose	277	219	189	226	220

[a] Expressed as nanomoles of O_2 min^{-1} mg of $protein^{-1}$ and corrected for endogenous oxygen uptake (14 to 15 nmol of O_2 min^{-1} mg of $protein^{-1}$). Cells were grown on 10 mM glucose and induced with the indicated carbon source. Data are averages of at least three independent determinations.

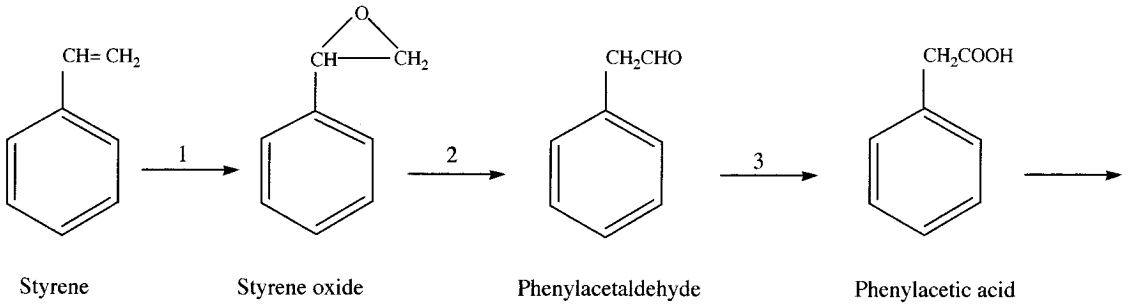

FIG. 2. Pathway for styrene degradation by *P. putida* CA-3 cells. 1, SMO; 2, SOI; 3, phenylacetaldehyde dehydrogenase.

Induction studies. Washed cell suspensions of *P. putida* CA-3 cells grown on glucose and induced by styrene or intermediates of styrene degradation oxidized styrene to various degrees (Fig. 1) (Table 3). Glucose-grown cells failed to oxidize styrene and intermediates of styrene degradation (Table 3). Styrene induced its own degradation and the degradation of intermediates subsequent to it in the pathway (Fig. 2). Styrene oxide and phenylacetaldehyde induced the degradation of styrene and all intermediates of styrene degradation. Phenylacetic acid-induced cells did not oxidize styrene or styrene oxide, but they did oxidize phenylacetic acid and phenylacetaldehyde (Table 3).

Repression of styrene degradation. *P. putida* CA-3 cells grown in MS medium which contained styrene and either glutamate or citrate illustrated the classical diauxic growth pattern (Fig. 3). Respiration studies performed with washed whole cells grown in the presence of styrene and either citrate or glutamate revealed that styrene degradation was repressed in the presence of glutamate and citrate (Table 4) (Fig. 3A and B). This repression was maintained as long as the repressing carbon source was present in the medium. Cells grown under the same repressing conditions in the presence of phenylacetic acid and glutamate (Table 4) (Fig. 3C) and citrate (data not shown) also failed to oxidize phenylacetic acid. Low-level oxidation of styrene and phenylacetic acid was seen in the diauxic parts of respective growth curves (Table 4). The level of styrene and phenylacetic acid oxidation increased significantly in the mid-log phase of growth on styrene or phenylacetic acid when the repressing carbon source had been depleted (Table 4). This diauxic growth pattern was not seen for cells grown on glucose and styrene (Fig. 1). Similarly, *P. putida* CA-3 cells grown on succinate and styrene or acetate and styrene did not show the classic diauxic pattern of growth (data not shown).

DISCUSSION

The presence of both SMO and SOI activities together with the inability of *P. putida* CA-3 cells to grow on carbon sources such as propyl-, butyl-, and ethylbenzene and benzene indicates that the initial attack on styrene is via the side chain and is not directly on the aromatic nucleus.

The observed pH-dependent shift in the absorbance maxima of the accumulating yellow substance in media of stationary-phase phenylacetic acid-grown cells has previously been reported to be indicative of the formation of *meta* cleavage products (4). This suggests that the styrene-degradative pathway proceeds via *meta* cleavage.

On the basis of enzyme activities (Table 2) and growth substrate utilization patterns (Table 3), a styrene-degradative pathway (Fig. 2) appears to operate in *P. putida* CA-3. Previous studies of bacterial styrene metabolism have proposed pathways similar to that which exists in this strain. Shirai and Hisatsuka proposed styrene oxide as an intermediate of styrene degradation (16). Hartmans et al. showed that degradation of styrene oxide and styrene in two different species of bacteria proceeded via phenylacetic acid (8). The first step in our pathway is oxygen-dependent epoxidation of styrene to styrene oxide, which is subsequently isomerized to phenylacetaldehyde. Phenylacetaldehyde is then oxidized to phenylacetic acid, which eventually enters the tricarboxylic acid cycle.

Enzyme activities in crude cell extracts of *P. putida* CA-3 (Table 2) show that PMS-dependent phenylacetaldehyde dehydrogenase activity is not significantly stimulated by NH_4Cl. This finding differs from that of Duine and coworkers for a quinoprotein methanol dehydrogenase (5) and from that observed by Hartmans and coworkers in *Xanthobacter* strain 124X for phenylacetaldehyde dehydrogenase activity (7). Styrene-, styrene oxide-, and phenylacetaldehyde-grown *P. putida* CA-3 cells have approximately the same relative levels of PMS- and NAD^+-dependent phenylacetaldehyde dehydrogenase activities. Extracts of *P. putida* CA-3 cells grown on phenylacetic acid possess high-level $NADP^+$-dependent and low-level NAD^+- and PMS-dependent phenylacetaldehyde dehydrogenase activities (Table 2). The SOI and phenylacetaldehyde dehydrogenase activities of styrene-, styrene oxide-, and phenylacetaldehyde-grown *P. putida* CA-3 cells are at the same relative levels, suggesting a coordinately regulated system of induction for the enzymes involved in styrene oxide degradation and phenylacetaldehyde degradation (Table 2).

Styrene induces all of the enzymes that convert styrene to phenylacetic acid in *P. putida* CA-3 cells (Table 3). Thus, styrene, like toluene, appears to activate the catabolism of all intermediates of its own degradation (1, 6). Styrene oxide and phenylacetaldehyde act to induce the degradation of themselves and aromatic compounds that precede them in the pathway. Phenylacetic acid-induced cells fail to oxidize styrene and styrene oxide (Table 3), and extracts of *P. putida* CA-3 cells grown on phenylacetic acid do not possess detectable SOI activity (Table 2). However, phenylacetic acid-induced cells do oxidize phenylacetic acid and phenylacetaldehyde (Table 3). This observation together with the presence of $NADP^+$-dependent phenylacetaldehyde dehydrogenase activity (Table 2) in phenylacetic acid-grown cells suggests the presence of a second enzyme activity whose induction is regulated by phenylacetic acid, not phenylacetaldehyde.

Therefore, there appears to be a division in the styrene-degradative pathway of *P. putida* CA-3 similar to that which has been described for toluene degradation in other *Pseudomonas* strains (1, 6). Styrene, styrene oxide, and phenylacetaldehyde are upper pathway intermediates and induce the degradation of all the intermediates tested, whereas phenylacetic

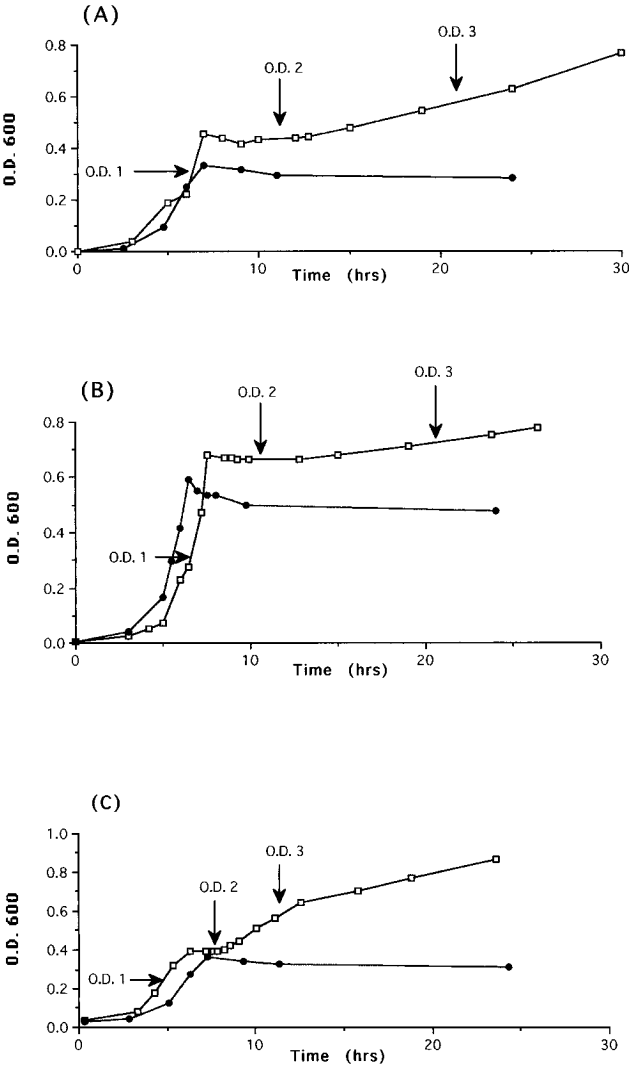

FIG. 3. Catabolite repression by citrate and glutamate of styrene degradation by *P. putida* CA-3. Cells were harvested for substrate utilization analysis at the OD_{600} points indicated by arrows. Growth of *P. putida* CA-3 cells occurred in MS media which contained 0.1% citrate (●) or 0.1% citrate and styrene (□) (A), 0.07% glutamate (●) or 0.07% glutamate and styrene (□) (B), or 0.1% glutamate (●) or 0.1% glutamate and 0.1% phenylacetic acid (□) (C).

acid is a lower pathway intermediate and induces the degradation of only itself and phenylacetaldehyde. The induction of $NADP^+$-dependent phenylacetaldehyde dehydrogenase activity in phenylacetic acid-induced cells (Table 2 [66 nmol]) is probably responsible for the increases in oxygen uptake rates when phenylacetic acid-induced cells oxidize phenylacetaldehyde (Table 3 [315 nmol]).

Carbon source-dependent repression in *Pseudomonas* species has previously been reported to affect the catabolism of aromatic carbon sources, such as toluene (9) and ethylbenzene (3), and the activity of protocatechuate 3,4-dioxygenase (19). An organic acid or a tricarboxylic acid cycle intermediate, not glucose, is usually the preferred carbon source in *Pseudomonas* species (11). *P. putida* CA-3 preferentially utilizes glutamate and citrate before styrene when they coexist in the same medium, as seen in the substrate utilization experiments (Table 4) (Fig. 3A and B). The regulation of styrene degradation by glutamate and citrate in strain CA-3 appears to be similar to

TABLE 4. Carbon source-dependent oxidation of styrene by *P. putida* CA-3 cells

Substrate	Rate of oxygen uptake at specific OD_{600} by cells grown on[a]:								
	Glutamate and styrene			Citrate and styrene			Glutamate and phenylacetic acid		
	OD 1	OD 2	OD 3	OD 1	OD 2	OD 3	OD 1	OD 2	OD 3
Styrene	<5	18	109	<5	20	103	—[b]	—	—
Glutamate	41	28	24	—	—	—	86	46	43
Citrate	—	—	—	79	45	21	—	—	—
Phenylacetic acid	—	—	—	—	—	—	<5	171	395

[a] Expressed as nanomoles of O_2 min^{-1} mg of $protein^{-1}$ and corrected for endogenous oxygen (15 to 20 nmol of O_2 min^{-1} mg of $protein^{-1}$). Data are averages of at least three independent determinations. OD 1, -2, and -3, ODs of cultures at the time of harvesting (Fig. 3).

[b] —, not determined.

the control exerted on nonaromatic carbon sources such as histidine (14) and glucose (12) in other *Pseudomonas* species. While the styrene-degradative pathway appears to be divided into an upper regulon and a lower regulon, the enzymes for both parts of this pathway appear to be repressed in similar fashion as glutamate and citrate also repress the degradation of phenylacetic acid (Table 4).

The genes that encode both SMO and SOI activities in *P. putida* CA-3 have previously been cloned (13). This provides us with a tool to study the molecular mechanism of induction and repression of styrene degradation in our strain.

In summary, the styrene-degradative pathway in *P. putida* CA-3 appears to be divided into an upper pathway that consists of styrene, styrene oxide, and phenylacetaldehyde and a lower pathway that begins with phenylacetic acid. Styrene metabolism is induced by styrene and products of styrene degradation, such as styrene oxide and phenylacetaldehyde, and repressed in the presence of nonaromatic carbon sources, such as glutamate and citrate.

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