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Catabolite-Mediated Mutations in Alternate Toluene Degradative Pathways in *Pseudomonas putida*

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***Pseudomonas putida* 54g grew on mineral salts with toluene and exhibited catechol-2,3-dioxygenase (C23O) activity, indicating a *meta* pathway. After 10 to 15 days on toluene, nondegrading (Tol$^-$) variants approached nearly 10% of total CFU. Auxotrophs were not detected among variants, suggesting selective loss of catabolic function(s). Variant formation was substrate dependent, since Tol$^-$ cells were observed on neither ethylbenzene, glucose, nor peptone-based media nor when toluene catabolism was suppressed by glucose. Unlike wild-type cells, variants did not grow on gasoline, toluene, benzene, ethylbenzene, benzoate, or catechol, suggesting loss of *meta* pathway function. Catabolic and C23O activities were restored to variants via transfer of a 78-mDa TOL-like plasmid from a wild-type Tol$^+$ donor. Tests for reversion of variants to Tol$^+$ were uniformly negative, suggesting possible deletion or excision of catabolic genes. Deletions were confirmed in some variants by failure to hybridize with a DNA probe specific for the *xylE* gene encoding C23O. Cells grown on benzoate remained Tol$^+$ but were C23O$^-$ and contained a plasmid of reduced size or were plasmid free, suggesting an alternate chromosomal catabolic pathway, also defective in variants. Cells exposed to benzyl alcohol, the initial oxidation product of toluene, accumulated >13% variants in 5 days, even when cell division was repressed by nitrogen deprivation to abrogate selection processes. No variants formed in identical ethylbenzene-exposed controls. The results suggest that benzyl alcohol mediates irreversible defects in both a plasmid-associated *meta* pathway and an alternate chromosomal pathway.**

Hydrocarbon-degrading bacteria often display remarkable catabolic diversity (1, 3, 10, 11, 13, 15, 33); however, they may lose catabolic activity during subculture, even if the hydrocarbon is provided as the sole source of carbon and energy (5, 22, 41, 46–48). Loss of degradative function can be unpredictable and irreversible, which may be problematic in genetic studies (5, 6) and bioremediation applications (8, 19).

The best-understood catabolic pathway in terms of regulation and stability is encoded by the archetypal TOL plasmid pWWO of *Pseudomonas putida* MT-2 (4, 6, 10, 12–15, 46). The TOL plasmid is 117 kb, about 40 kb of which are required for the known catabolic and regulatory genes (4, 12–15). The plasmid consists of two overlapping transposons (7, 42–44) encoding biotransformation of toluene and *m*/*p*-xylene via *meta* cleavage of the aromatic ring by catechol-2,3-dioxygenase (C23O) (4, 15, 20, 26, 27). Plasmid-free *P. putida*, or strains harboring defective plasmids, may still grow on toluene without C23O activity (4, 28, 35, 41, 47) via a chromosomal *ortho* cleavage pathway (4, 16, 30, 50). Chromosomal pathways exhibit partial homology with pWWO but are regulated differently and prone to deletions and rearrangements (30, 50).

Interestingly, TOL plasmids are most stable on nonselective substrates, such as succinate (22), propionate (47), or gluconate (41), in the absence of catabolic gene induction and expression. Accordingly, Jain et al. (18) demonstrated that pWWO was stably maintained in *P. putida* MT-2 for prolonged incubations (>8 weeks) in native groundwater microcosms in the absence of toluene or other pathway inducers. It has been suggested that plasmid replication may not be a disadvantage to the cell, at least under certain nutritional conditions, but that gene expression may be (22).

In contrast, pWWO and related TOL plasmids undergo deletion or curing on benzoate (2, 4, 6, 13, 20, 21, 23, 25, 28, 29, 47, 48). Chakrabarty (6) reported a 28-mDa excision from an RP4::TOL cointegrate (pAC8) following growth of *P. putida* MT-2 on benzoate. Excision was dependent on host *recA* function, consistent with directly repeated 1.4-kb sequences flanking the TOL catabolic region (25). Certain regions of TOL can be transposed to the chromosome (13, 37) or nonhomologous target replicons independent of host *recA* function (7, 13, 36, 42–44), and several classes of deletion mutants have been identified in *P. putida* MT-14, MT-15, and MT-20 following growth on benzoate (13, 21, 23, 29, 41, 48). Carney and Leary (5) reported substrate-dependent gene rearrangements and chromosomal insertions associated with a 95-kb TOL-like plasmid in *P. putida* R5-3.

Whether benzoate acts directly to invoke TOL excisions (e.g., by transposase activation) or simply favors selection of preexisting (spontaneous) deletion mutants is unresolved. Williams et al. (47) proposed that benzoate curing or deletion of pWWO in *P. putida* MT-2 occurs spontaneously and that mutants outcompete wild-type cells because of a growth advantage on benzoate. However, a direct effect of benzoate on catabolic gene stability was not ruled out, and benzoate catabolism was required for curing or deletion of pWWO carrying Tn*401* insertions.

Keshavarz et al. (22) suggested that benzoate interferes with plasmid partitioning after observing loss and recovery of a nonconjugative TOL plasmid in *P. putida* PPK1 grown in continuous culture with benzoate or *m*-toluate, respectively. A similar hypothesis was proposed by Stephens and Dalton (41), who observed TOL deletions and loss in *P. putida* MT-15 grown on undissociated benzoate and related lipophilic com-

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pounds (e.g., *m*-toluate, acetate, butyrate, and butanol) suspected of interacting with cell membranes.

Saint and Venables (35) reported that growth of *P. putida* UCC22 (an MT-2 derivative) on benzoate, and to a lesser extent on succinate, glucose, or acetate, resulted in deletion or curing of plasmid pTDN1 encoding aromatic amine and *m*-toluate degradation. Deletions resulted from a recombinational excision at 2-kb direct repeats flanking the 28-kb catabolic sequences, similar to TOL; yet, pTDN1 shared little homology with pWWO, making generalization difficult (34). A proportional increase of mutants was not attributed to an effect of benzoate on the plasmid or partitioning but to a growth advantage of mutants.

Recently, high frequencies of stable, toluene-nondegrading mutants (Tol^- variants) were observed in batch cultures of *P. putida* 54g grown in mineral salts with toluene or gasoline. Since this environmental isolate was being used in bioreactor studies, loss of its degradative phenotype was of special concern. Experiments designed to reveal the nature and origin of Tol^- variants in *P. putida* 54g are described.

MATERIALS AND METHODS

Bacterial strains and media. *P. putida* 54g was isolated from a gasoline-contaminated aquifer in Seal Beach, Calif. (33). *Escherichia coli* LE392-1 harboring plasmid RP4 was provided by M. Silverman, Agouron Institute, La Jolla, Calif. *P. putida* MT-2 harboring pWWO was obtained from the American Type Culture Collection, Rockville, Md. (ATCC 33015). *P. putida* PWWO was provided by the late J. Leary, Department of Biology, University of California, Riverside. *Pseudomonas* strains were grown on HCMM2 mineral salts with unleaded gasoline (5 to 20% toluene by volume), toluene, or other hydrocarbon vapors in glass-Teflon containers (e.g., HCMM2-toluene [33]). Spectrophotometric-grade hydrocarbons were obtained from Aldrich Chemical Co., Milwaukee, Wis., except for benzyl alcohol and benzaldehyde, which were obtained from Sigma Chemical Co., St. Louis, Mo. Tol^- variants were maintained on HCMM2-glucose or R2A (Difco Laboratories, Detroit, Mich.), a yeast extract-peptone medium for heterotrophic bacteria (32).

Detection and enumeration of Tol^- variants. In one method, about 600 colonies were picked from R2A to HCMM2-toluene and R2A. Transferred cells forming colonies on R2A but not on HCMM2-toluene within 10 days at 23°C were regarded as putative variants. These were retested for growth on toluene, other hydrocarbons, intermediates, and glucose (to check for auxotrophs). Cells which grew on glucose but not hydrocarbon and which failed to revert to toluene utilization (Tol^+) were regarded as confirmed Tol^- variants. The detection limit was about 1 cell per 600.

In the second method, variants were enumerated by direct selection on R2A containing 500 μg of nalidixic acid per ml ($R2A+Nal_{500}$), a DNA gyrase inhibitor (39). The detection limit was about 1 cell per 10^{10}. A small proportion of nalidixic acid-resistant (Nal^r) colonies (typically <2%) were Tol^+; thus, it was necessary to routinely screen samples of Nal^r colonies for growth on HCMM2-toluene.

Variant reversion tests. Reversion of Tol^- to Tol^+ was tested by growing variants in 5.0 ml of R2A broth for 18 h at 28°C to approximately 10^{11} cells/ml, determined by epifluorescence microscopy with the fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) (9, 31). Cells were pelleted (Beckman JA-20 rotor; 7,000 rpm, 10°C, 10 min), washed twice, and resuspended in 5.0 ml of HCMM2. Aliquots were plated on (i) R2A to enumerate total CFUs and (ii) HCMM2-toluene. Absence of colonies after 2 weeks on HCMM2-toluene indicated lack of reversion to Tol^+. The reversion detection limit was about 1 cell per 10^{10} CFU.

Nitrogen limitation with toluene. Wild-type strain 54g was grown in HCMM2-toluene broth for 24 h at 23°C. Cells were harvested and washed twice by centrifugation; one portion was resuspended in HCMM2-toluene lacking nitrogen (N-free medium), while another was resuspended in complete HCMM2-toluene. Flasks were incubated at 23°C, and at intervals, Tol^- cells were determined. A variation of this experiment involved growing strain 54g colonies on 47-mm cellulose acetate filters (0.45-μm pore size; Millipore Corp., Bedford, Mass.) at 23°C on HCMM2-toluene plates. After 48 h, while variant formation was still low or undetectable, one filter set was transferred to N-free toluene and another was transferred to complete HCMM2-toluene. Colonies were picked at intervals, and variants were determined.

N limitation with benzyl alcohol. Wild-type cells pregrown in HCMM2-ethylbenzene for 24 h at 23°C to ensure absence of detectable Tol^- variants (see Results) were harvested by centrifugation, washed twice, resuspended in N-free HCMM2, and then starved for several hours at 28°C. Starved cells were immobilized on 47-mm cellulose acetate filters (0.2-μm pore size; Millipore) that were prewashed with N-free HCMM2. Filters were placed on N-free HCMM2 plates, incubated in ethylbenzene or benzyl alcohol vapors for 5 days at 23°C, transferred to R2A and $R2A+Nal_{500}$ plates, and incubated for up to 2 weeks at 23°C. The proportions of Tol^- colonies on both media were then enumerated. Identically prepared filters (0.2-μm pore size; polycarbonate; Costar, Pleasanton, Calif.) were used to enumerate N-deprived (DAPI-stained) cells per microcolony before and after hydrocarbon exposure by epifluorescence microscopy coupled with digital image analysis.

Determination of C23O activity. C23O activity was determined by the catechol oxidation test (5, 35, 41). Positive (toluene-grown *P. putida* MT-2) and negative (glucose-grown *P. putida* 54g) controls were tested routinely with experimental plates. The colony tests were sometimes performed in parallel with enzyme assays on crude cell lysates (5, 45), which yielded similar results.

DNA extractions. DNA was extracted by a modification of Maniatis et al. (24). Cells grown for 48 h in 150 ml of HCMM2-toluene (or R2A for variants) were harvested by centrifugation and washed in ice-cold 20% (wt/vol) sucrose in 50 mM Tris-HCl (pH 8.0; Tris buffer). Cell lysis was for 10 min at 4°C with lysozyme (20 mg/ml in 0.50 M Tris [pH 8.0]) and 20% (wt/vol) sodium dodecyl sulfate. DNA suspensions were extracted twice with phenol-chloroform (2:1) and once with absolute chloroform, and the aqueous phase was incubated overnight in 70% ethanol at −20°C.

Agarose gel electrophoresis. DNA samples were analyzed by agarose gel electrophoresis in Tris-borate buffer (89 mM Tris, 2.5 mM disodium EDTA, 89 mM boric acid [pH 8.2]). Gels contained 0.7% SeaKem Gold agarose (FMC Bioproducts, Rockland, Maine), and electrophoresis was at 90 V (4.1 V/cm) for 16 h with a model PP1-200 field-reversal electrophoresis controller (MJ Research, Watertown, Mass.). Gels were stained with 0.5 μg of ethidium bromide per ml and visualized as described by Maniatis et al. (24).

DNA hybridizations. Total cell DNA (extracted as described above) was hybridized to an 18-bp DNA oligomer specific for the primer region of the *xylE* gene. The probe sequence was 5′ CTA TGA AGA GGT GAC GTC 3′ (27). The oligomer was synthesized on a model 1000 DNA synthesizer (Beckman Instruments, Fullerton, Calif.) and 3′ labeled by direct conjugation of alkaline phosphatase to an amino-modified nucleotide (Lightsmith II luminescence engineering system for oligonucleotides; Promega, Madison, Wis.). Concentration of the synthetic oligonucleotide was determined by UV absorption at 260 nm. Hybridization was performed for 45 min at 45°C at a final probe concentration of 250 fmol/ml by a modified Southern blot (40) with nylon membranes (Boehringer Mannheim, Indianapolis, Ind.). Membranes were autographed with Kodak XAR-5 film (Sigma) at 23°C for 2 h.

Conjugation experiments. A Nal^r Tol^- variant was conjugated with a Nal^s RP4::TOL wild-type *P. putida* donor. The donor was prepared by mating wild-type strain 54g with *E. coli* LE392-1 carrying RP4 resistant to tetracycline (Tc^r). Tol^+ Tc^r Nal^r transconjugants were selected on HCMM2-toluene supplemented with either tetracycline (50 μg/ml) or nalidixic acid (500 μg/ml).

RESULTS AND DISCUSSION

Properties of wild-type strain 54g. *P. putida* 54g exhibited growth and C23O activity on HCMM2 mineral salts supplemented with gasoline, toluene, benzene, or ethylbenzene (Table 1). Growth on the intermediates benzyl alcohol, benzaldehyde, benzoate, and catechol with C23O activity suggested that toluene was catabolized via a *meta* pathway similar or identical to that of *P. putida* MT-2 harboring pWWO (4, 13) (Fig. 1). Wild-type 54g cells contained a single 118-kb (78-mDa) TOL-like DNA plasmid indistinguishable in agarose gels from the pWWO plasmid of *P. putida* MT-2 (Fig. 2). The Tol^+ phenotype could be restored to Tol^- variants by RP4-assisted mobilization of this TOL-like plasmid (see Materials and Methods). The plasmid was transferred to a Nal^r Tol^- variant at a frequency of 2.50×10^{-5} and restored both C23O activity and the ability to grow on HCMM2-toluene in the presence of 500 μg of nalidixic acid per ml or 50 μg of tetracycline per ml.

Formation of Tol^- variants. Incubation of *P. putida* 54g (or MT-2; data not shown) in HCMM2 batch culture with toluene or gasoline vapors resulted in a proportional increase of Tol^- variants (Fig. 3), which could no longer grow on toluene or other hydrocarbons (see below). The proportion of variants typically approached 10% of total (R2A) CFU within 10 to 15 days. As indicated in Fig. 3, the proportional increase in Tol^- cells resulted from a combination of loss of wild-type cells and an actual increase in variant numbers after cultures entered the stationary phase, suggesting continued variant formation and/or a growth advantage. As the culture aged, Tol^- and wild-type

TABLE 1. Properties of *P. putida* 54g strains used in this study

Characteristic	Tol^+ wild type	Tol^- variants[a]	Benzoate-grown Tol^+	Tol^+ plasmid free[b]
Growth on:				
Gasoline	+	[c]−	+	+
Toluene	+	−	+	+
Ethylbenzene	+	−	+	+
Benzene	+	−	+	+
Benzyl alcohol	+	−	+	−
Benzaldehyde	+	−	+	+
Benzoate	+	−	+	+
Catechol	+	−	+	+
Glucose	+	+	+	+
R2A medium	+	+	+	+
C23O activity	+	ND[c]	−	−
TOL-like plasmid[d]	+	±	±	−
Chromosomal pathway[e]	+	−	+	+
Nalidixic acid resistance[f]	S	R	S	S

[a] Substrate growth tests based on analysis of >200 different Tol^- variants.
[b] Plasmid-free strains obtained following growth of wild-type 54g on HCMM2-benzoate (see Results).
[c] ND, test not done, but growth on catechol was negative.
[d] ±, TOL-like plasmid may or may not be present.
[e] +, presence of functional chromosomal toluene degradative pathway permitting growth on toluene without C23O activity.
[f] S, sensitive to 50 μg of nalidixic acid per ml in R2A; R, resistant to 500 μg of nalidixic acid per ml in R2A.

cells codeclined in response to adverse (toxic or starvation) conditions.

Properties of Tol^- variants. Variants were unable to grow on any hydrocarbons or intermediates tested (Table 1), suggesting that required catabolic enzymes were defective or absent in these strains. Lack of growth on hydrocarbons suggested that variants multiplied at the expense of organics leaked from wild-type cells, a process expected to be greatest in stationary or decline-phase cultures. Of >1,000 variants tested, none spontaneously reverted to Tol^+ at frequencies greater than 10^{-10}, and they all grew on HCMM2-glucose, indicating that essential biosynthetic pathways were not mutated to auxotrophy at rates comparable to those of loss of the Tol^+ phenotype (Table 1).

Three variant categories were observed routinely. One harbored plasmids of unchanged molecular weight, although small deletions or excisions would have been overlooked in gels (Fig. 2). A second variant category harbored a TOL-like plasmid of reduced size (data not shown), suggesting deleted or excised sequences, a possibility considered likely given the lack of reversion of variants to Tol^+. A third category lacked demonstrable plasmid DNA (cured).

Confirmed Tol^- variants were uniformly resistant to 500 μg of nalidixic acid per ml (Table 1), whereas wild-type cells were Nal^s at <50 μg/ml. The Nal^r trait allowed the direct selection and conservative enumeration of Tol^- variants (see Materials and Methods). The mechanism of acquisition of Nal^r by variants was not investigated in this study; however, the existence of plasmid-free Nal^r variants suggested that a gene(s) encoding antibiotic resistance was chromosomal. Tol^+ transconjugants derived from mating experiments (see above) remained Nal^r, suggesting no repression of this host genomic marker by a functional plasmid.

Some Tol^- variants contain deletions. The absence of reversion of Tol^- cells to Tol^+ suggested that catabolic gene sequences were deleted in variants. Deletions were confirmed in total DNA extracts from some variants (e.g., strain v28, Fig. 4) by lack of hybridization with a DNA probe specific for the *xylE* gene encoding C23O. Other Tol^- variants probed positive for *xylE* (strains v10 and v29) suggesting that different sequences were deleted or otherwise irreversibly altered in different variants. Finally, some Tol^- variants which lacked the 78 MDa plasmid still probed positive for *xylE* (e.g., strain v33, Fig. 2 and 4), suggesting possible chromosomal integration of *xylE* similar to that described by Carney and Leary for *P. putida* R5-3A (5). These data indicate there is more than one mechanism of variant formation in strain 54g.

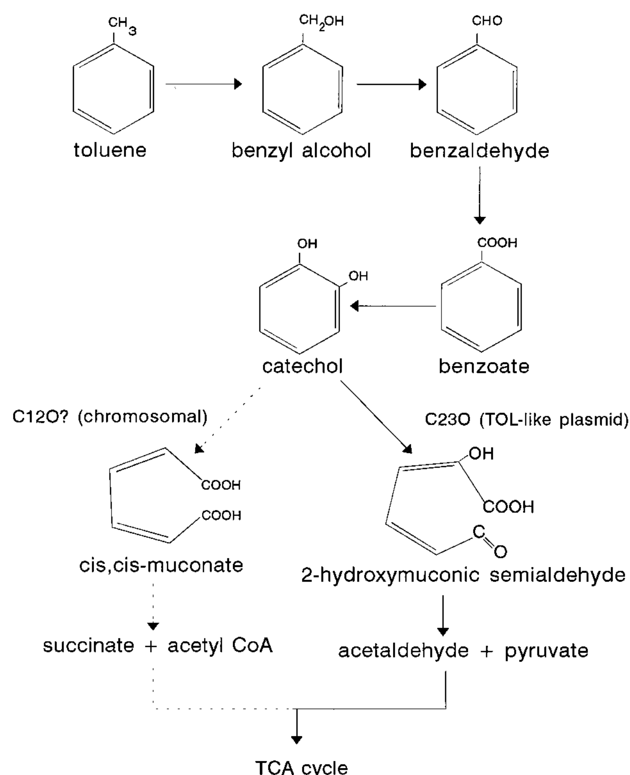

FIG. 1. Toluene catabolic pathways in *P. putida* 54g. The *meta* pathway is encoded by a 78-mDa TOL-like plasmid, but the nature of the alternate chromosomal pathway is still unknown. TCA, tricarboxylic acid.

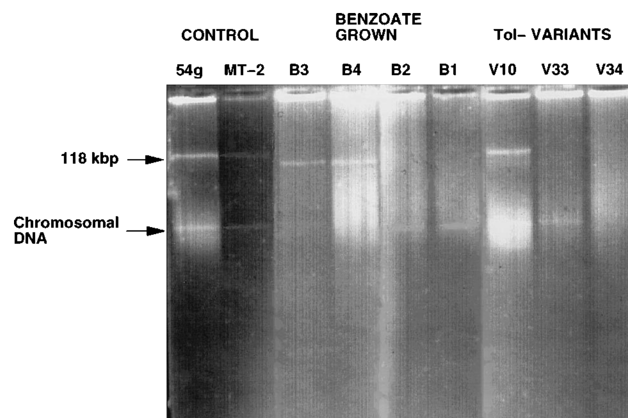

FIG. 2. Agarose gel electrophoresis of plasmid DNA from wild-type *P. putida* 54g grown on HCMM2-toluene (54g), strain MT-2 grown on HCMM2-toluene (MT-2), wild-type 54g grown on HCMM2-benzoate (B1 to B4), and Tol^- variants (V10, V33, and V34) grown on R2A.

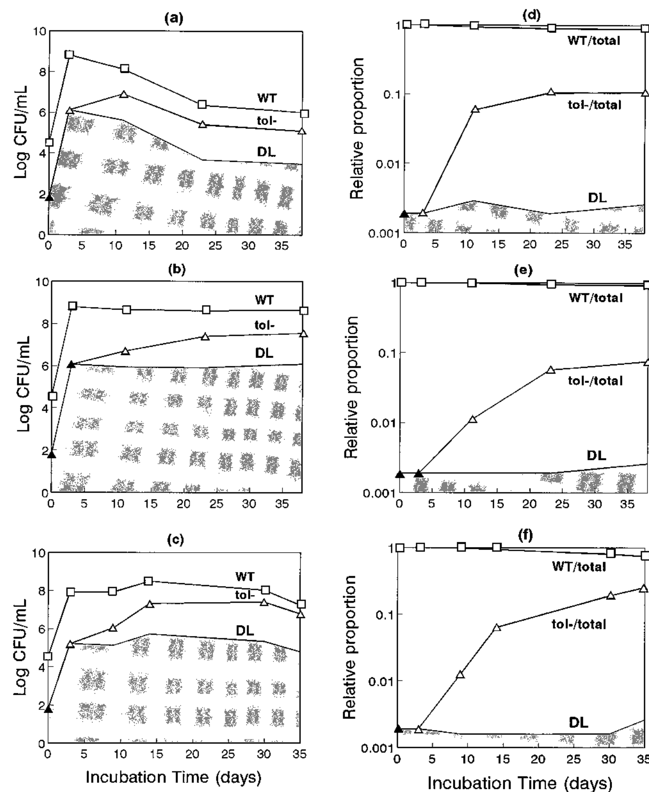

FIG. 3. Numbers (a to c) and corresponding proportions (d to f) of wild-type (WT) and Tol$^-$ cells as a function of batch culture age. Broth media were HCMM2-gasoline (a and d), HCMM2-toluene (b and e), and HCMM2-benzyl alcohol (c and f). DL, variant detection limit (see Materials and Methods). Filled symbols indicate that variants were assayed but below the detection limit.

Tol$^-$ variant formation is substrate dependent. Tol$^-$ variants were not observed on ethylbenzene, glucose, or R2A, regardless of lengthy incubations (to 40 days), indicating that variant production was substrate dependent (Table 2). Wild-type cells grown on R2A or HCMM2-glucose in the presence of gasoline or toluene lacked C23O activity (because of glucose repression of *meta* pathway promoters [17]) and did not produce Tol$^-$ variants (Table 2). Thus, native untransformed hydrocarbons were not responsible for variant formation. Accordingly, a greatly reduced proportion of Tol$^-$ variants (<0.1%) was observed when log-phase cells (pregrown on HCMM2-toluene) were resuspended in N-free HCMM2-toluene to retard growth and catabolic activity (Table 2). R2A plate counts indicated that cells in N-free medium did not multiply significantly over a 21-day incubation. Additionally, O_2-electrode measurements indicated that the rate of respiration of cells in N-free HCMM2-toluene decreased to about 0.1 of controls in 24 h (data not shown); thus, toluene catabolism and variant formation were suppressed under N-limited conditions.

Tol$^-$ variants are not formed on benzoate. The observations described above suggested that a toluene catabolic pathway(s) must be induced and toluene must be degraded as a prerequisite to Tol$^-$ variant formation. However, growth of *P. putida* 54g on benzoate failed to produce Tol$^-$ variants as defined in this study (Tables 1 and 2). Instead, benzoate-grown cells remained Tol$^+$ but uniformly lacked C23O activity (C23O$^-$), suggesting both loss or inactivation of the plasmid-associated *meta* pathway and operation of an alternate toluene degradative pathway not susceptible to benzoate but still missing or defective in variants.

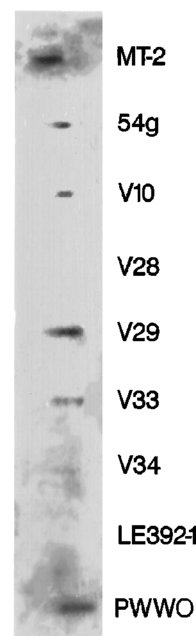

FIG. 4. Southern blot analysis of total cellular (plasmid plus chromosomal) DNA from *P. putida* MT-2, wild-type 54g, PWWO, Tol$^-$ variants (V10, V28, V29, V33, V34) and *E. coli* LE392-1. The probe was specific for *xylE* (see Materials and Methods).

Isolation of plasmid-free strains following growth on benzoate. Evidence that the alternate pathway was chromosomally encoded was obtained by isolation of Tol$^+$ C23O$^-$ plasmid-free strains of *P. putida* 54g following growth on HCMM2-benzoate (0.5% [wt/vol]) at pH 6.5 (Fig. 2; Table 1). Cured Tol$^+$ strains remained Nal^s; thus, acquisition of antibiotic resistance was associated with loss of chromosomal pathway function. Interestingly, when plasmid-free strains were grown

TABLE 2. Influence of growth substrate on Tol$^-$ variant formation in *P. putida* 54g

Growth substrate[a]	Tol$^-$ variants observed	Frequency in cultures (%)[b]
Gasoline[c]	Yes	1–10
Toluene	Yes	1–10
Ethylbenzene	No	ND[d]
Benzylalcohol	Yes	5–20
Benzaldehyde	Yes	<0.01
Benzoate	No[e]	ND
Catechol	No	ND
Glucose	No	ND
R2A medium	No	ND
Gasoline-glucose[c]	No	ND
Gasoline-R2A[c]	No	ND
Toluene-glucose	No	ND
Toluene, N-free[f]	Yes	<0.1
Glucose, N-free[f]	No	ND

[a] All hydrocarbons except benzoate were supplied as vapors.
[b] Typical proportion of R2A plate count as Tol$^-$ variants after 1 to 2 weeks in batch culture at 23°C.
[c] Unleaded gasoline.
[d] ND, no Tol$^-$ variants detected by toothpicking or nalidixic acid selection (see Materials and Methods).
[e] Cells are uniformly Tol$^+$, but C23O$^-$, after growth on HCMM2-benzoate, suggesting defective plasmid.
[f] See Materials and Methods for details.

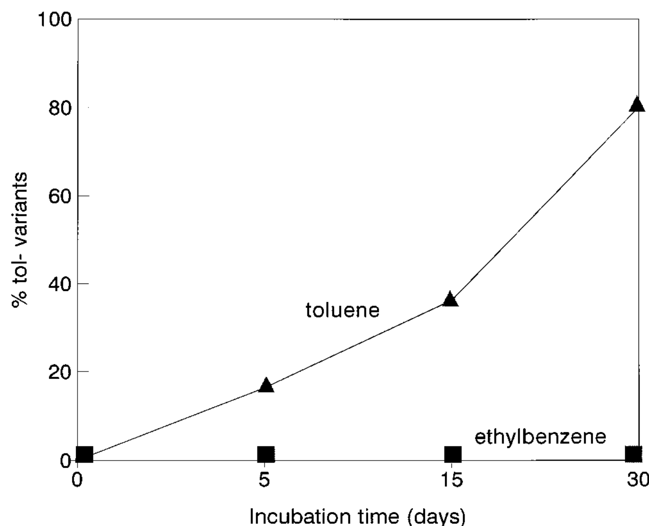

FIG. 5. Percentage of total CFUs as Tol$^-$ variants formed on HCMM2-toluene or HCMM2-ethylbenzene inoculated with a Tol$^+$ plasmid-free (C23O$^-$) strain (20B3). Strain 20B3 was isolated following growth of wild-type 54g on HCMM2-benzoate.

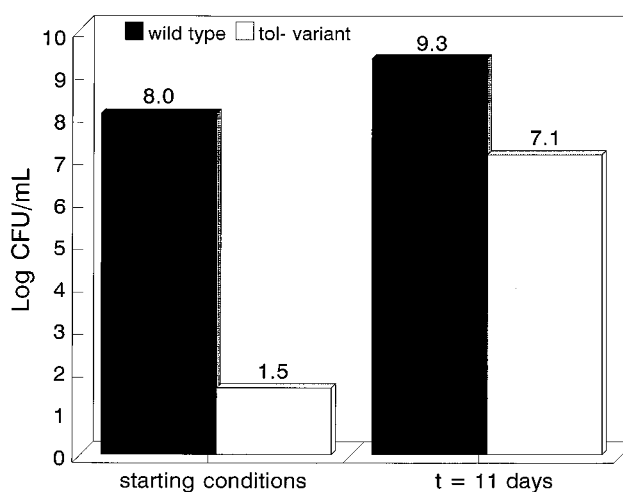

FIG. 6. Proportional increase of a Nal^r Tol$^-$ variant (strain v45) seeded into a late-log-phase wild-type 54g culture grown on HCMM2-ethylbenzene. Wild-type cells were Nal^s.

on HCMM2-toluene (but not on ethylbenzene), an unusually high proportion of Tol$^-$ variants was observed (Fig. 5), suggesting that the chromosomal pathway was especially unstable on toluene or its transformation product(s) or that variant formation is more likely with only one (chromosomal) pathway.

Benzyl alcohol promotes Tol$^-$ variant formation. Since Tol$^-$ cells were not observed on benzoate, downstream intermediates (e.g., catechol) were not responsible for variant formation. Indeed, no Tol$^-$ cells were observed on HCMM2-catechol (Table 2). However, growth of wild-type 54g on HCMM2-benzyl alcohol resulted in a marked proportional increase of Tol$^-$ variants (Fig. 3; Table 2). Typically, 10 to 20% of total (R2A) CFU were Tol$^-$ after 1 to 2 weeks on benzyl alcohol, but values as high as 60% have been observed. When a benzoate-grown Tol$^+$ strain still harboring a defective (i.e., C23O$^-$) TOL-like plasmid was grown on HCMM2-benzyl alcohol, >80% of total CFU were Tol$^-$ after 30 days, suggesting an enhanced instability of the chromosomal pathway on this intermediate or an increased probability of single pathway loss. Tol$^+$ C23O$^-$ plasmid-free cells (from benzoate) exhibited no visible growth on HCMM2-benzyl alcohol, suggesting that these cells rapidly became Tol$^-$ on this substrate before significant growth could occur. No variants were detected on HCMM2-benzaldehyde after a 3-week incubation; however, a low proportion of variants was detected (4.5×10^{-5}) via nalidixic acid selection after 35 days.

Tol$^-$ cells grow when introduced into wild-type cultures. Two hypotheses may explain the origin of Tol$^-$ variants in strain 54g. Variants may arise spontaneously at low, possibly undetectable, frequencies, and certain substrates (e.g., benzyl alcohol) could favor their selection and amplification relative to that of wild-type cells in a growing population. Alternatively, conversion (mutation) from Tol$^+$ to Tol$^-$ may be mediated by a specific metabolic intermediate and, unlike selection, would not necessarily depend on cell multiplication. To distinguish between these hypotheses, a low titer (about 36 cells per ml) of a Nal^r Tol$^-$ variant (strain v45) was introduced into a late-log-phase broth culture of wild-type 54g grown overnight at 23°C in HCMM2-ethylbenzene. As noted above, variants were never detected in ethylbenzene cultures (Table 2), suggesting that selective conditions favoring growth of Tol$^-$ cells were not established with this substrate. Results presented in Fig. 6 indicate that the introduced Nal^r variant underwent a >6-log increase in ethylbenzene cultures, presumably at the expense of trace organics leaked from wild-type cells growing on the parent hydrocarbon. Wild-type cells increased about 1.3 logs during the same period, suggesting that Tol$^-$ cells enjoyed a transient growth advantage. No variants were recovered from an unseeded control culture incubated under the same conditions (data not shown). Nal^r colonies recovered at each sample time from seeded wild-type cultures were uniformly Tol$^-$, indicating a lack of transfer of Nal^r genes to wild-type cells during incubation. Similar results were obtained when strain v45 (<24 cells per ml) was inoculated into wild-type 54g cultures growing on R2A medium or when variants were placed into filter-sterilized media from wild-type cultures (data not shown). Thus, if a Tol$^-$ variant had spontaneously formed in ethylbenzene or R2A cultures, it should have multiplied to levels sufficient for its detection. Since variants were not detected in these media, even following prolonged incubation (>30 days), their spontaneous formation is unlikely.

Benzyl alcohol mediates Tol$^-$ formation during repressed cell multiplication. If Tol$^-$ cells arise from conversion by a transformation product (e.g., benzyl alcohol), their numbers might increase in the presence of the metabolite regardless of whether cells are multiplying. In contrast, any mechanism involving selection of rare spontaneous mutants requires extensive cell division to achieve a proportional increase in mutants relative to that of wild-type cells. To differentiate between these mechanisms, Tol$^-$ variant formation was determined among growth-restricted (N-deprived) wild-type cells which were immobilized on filters and exposed to either ethylbenzene or benzyl alcohol vapors for 5 days (see Materials and Methods). At zero time ($t = 0$), the numbers of N-deprived cells per filter microcolony for ethylbenzene- and benzyl alcohol-exposed populations were (means ± standard deviations) 1.02 ± 0.147 ($n = 46$) and 1.05 ± 0.213 ($n = 43$), respectively, and at $t = 5$ days, they were 8.25 ± 9.84 and 6.38 ± 7.08, respectively. The corresponding numbers of cell divisions for the two populations were 3.03 ± 3.30 and 2.67 ± 2.82, respectively, with >95% of cells in both populations undergoing fewer than five divisions during hydrocarbon exposure (Fig. 7).

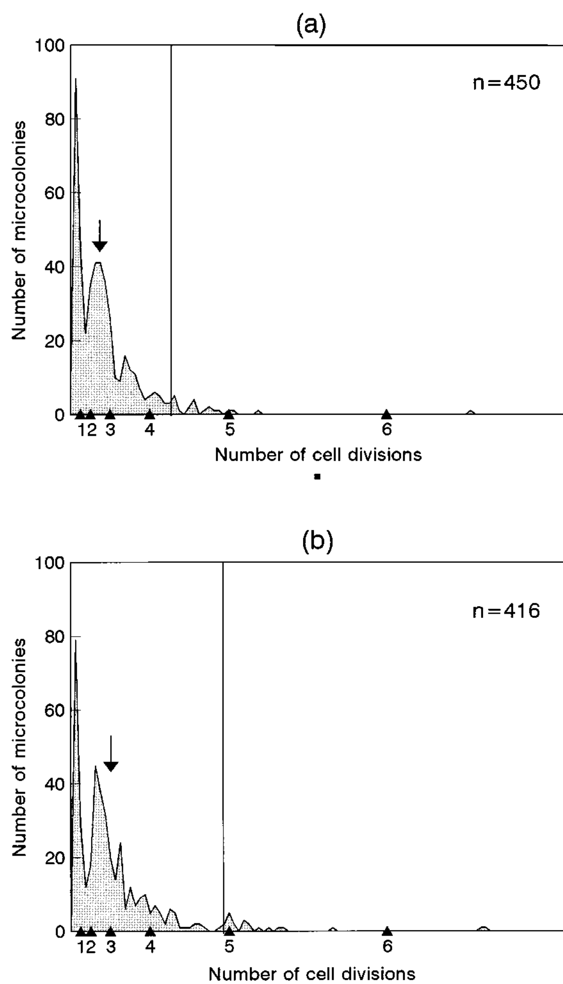

FIG. 7. Cell divisions occurring during 5-day exposure of identical filter populations to either benzyl alcohol (a) or ethylbenzene (b) vapors. Arrows indicate mean numbers of cell divisions; 95% of sample populations fall to the left of the vertical lines. Ordinates indicate actual microcolony numbers.

Wild-type cells for the experiment were pregrown in HCMM2-ethylbenzene and contained $<1.68 \times 10^{-9}$ variants determined by nalidixic acid selection. After hydrocarbon exposure, both filter sets were transferred to R2A and R2A+Nal_{500} to score Tol^- colonies via toothpicking and nalidixic acid selection, respectively. Of 200 R2A colonies with a history of benzyl alcohol exposure, 13% were confirmed as Tol^- variants. Similarly, 16% of the total viable (R2A) population was Tol^- by independent selection on R2A+Nal_{500}. In contrast, no Tol^- colonies were detected ($<1/10^6$ filtered cells) among the control population exposed to ethylbenzene.

Survival levels of N-deprived benzyl alcohol- and ethylbenzene-exposed populations after 5 days were 75.3 and 89.5%, respectively; thus, differential variant formation on these hydrocarbons was unrelated to population viability. In addition, nalidixic acid could not have caused variant formation in benzyl alcohol-exposed cells; otherwise, variants should have occurred in ethylbenzene controls where this antibiotic was also employed.

These data suggest that exposure of *P. putida* 54g to benzyl alcohol results in irreversible mutations in alternate toluene degradative pathways. In contrast, growth of wild-type 54g cells on benzoate results only in *meta* pathway defects (i.e., loss of C23O activity), but cells still grow on toluene via a chromosomal pathway. Tol^- variants formed at high frequencies (approximately 16%) among filter-immobilized wild-type cells exposed to benzyl alcohol (but not to ethylbenzene), even when cell division and selection processes were severely repressed as a result of imposed nitrogen limitation. Thus, benzyl alcohol is necessary for variant formation, and Tol^- cells accumulate under conditions where growth and selection processes are strongly repressed. Furthermore, the genetic defects in Tol^- cells occurred via a highly nonrandom mechanism, since nutritional auxotrophs were not detected among variants.

Since variants were not observed when wild-type cells were incubated under conditions of catabolic repression (i.e., in HCMM2-toluene with glucose or R2A supplementation), toluene degradative genes must be induced and toluene must be catabolized as a prerequisite for Tol^- variant formation. This conclusion is consistent not only with the lack of variant formation in cells grown on HCMM2-glucose, HCMM2-ethylbenzene, and R2A but also with reduced variant formation among cells transferred to N-free HCMM2-toluene, which repressed growth and substrate utilization. Keshavarz et al. (22) similarly reported that the TOL plasmid of *P. putida* PPK1 was most stable genetically when cells were maintained on succinate, i.e., in the absence of catabolic gene expression. A similar observation was made by Stephens and Dalton (41) for *P. putida* MT-15 growing on gluconate.

While the exact nature of gene defects in Tol^- variants is unclear, it is unlikely they involve simple base substitutions or other reversible phenomena. Transpositional rearrangements involving chromosomal integration of plasmid catabolic genes as described by Carney and Leary (5) for *P. putida* R3-5 growing on *m*-xylene or toluene may have occurred in strain 54g; however, unlike R5-3A, reversion to Tol^+ was not observed for strain 54g. The defects in strain 54g and the frequency with which they occur are more reminiscent of transposon-mediated excision mutations, similar to the HAA^- (halogenated alkanoic acid-negative) mutants reported in *P. putida* PP3 by Slater and coworkers (38). Indeed, lack of reversion of variants to Tol^+ and failure of the *xylE* probe to hybridize with one category of Tol^- mutants suggests that deletions or excisions occurred within the toluene degradative pathways in strain 54g.

Acquisition of nalidixic acid resistance was associated with loss of chromosomal (but not plasmid) catabolic function, since (i) plasmid-free Tol^- variants retained the Nal^r phenotype and (ii) Tol^+ $C23O^-$ benzoate-grown cells lacking a plasmid remained Nal^s. These observations are in accord with the known chromosomal location for this resistance marker in *P. putida* (16). Moreover, Tol^+ transconjugants from matings remained Nal^r, indicating no repression of Nal^r by the TOL-like plasmid. The mechanism of acquisition of nalidixic acid resistance is unknown, but the observations described above could be explained by excision of toluene catabolic sequences within or proximal to a chromosomal resistance gene, a possibility currently under investigation.

meta pathway defects (indicated by loss of C23O activity) in strain 54g following growth on benzoate was identical to that described for other *Pseudomonas* strains harboring pWWO or related TOL-like plasmids (13, 21, 23, 29, 41, 47) and might be expected to have a similar underlying mechanism. However, benzyl alcohol-mediated conversion of Tol^+ to Tol^- cells has not been reported, and neither the mechanism of action of benzyl alcohol nor the extent and nature of the genetic defects in variants have been elucidated. Benzoate and benzyl alcohol function differently in *P. putida* 54g, since the former resulted exclusively in *meta* pathway defects, whereas the latter pro-

duced coincident loss of both *meta* and chromosomal pathway functions. Comparison of restriction enzyme digests of Tol^+ $C23O^-$ cells harboring defective plasmids with benzyl alcohol-derived Tol^- variants may reveal whether similar or different regions of the TOL-like plasmid were affected.

Selective loss of a catabolic pathway is not necessarily lethal provided that an alternate carbon source is available to sustain viability and growth; the pathway could be restored under more favorable circumstances via genetic exchange (49). Nevertheless, irreversible loss of catabolic function in response to growth on its substrate seems counterproductive, and it is unclear why an organism would respond in this manner unless loss of this trait also increased organism fitness or survivability. Determination of the mechanism of benzyl alcohol-mediated loss of catabolic functions in *P. putida* 54g may help elucidate the reason for this phenomenon.

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