Mutants of *Pseudomonas cepacia* G4 Defective in Catabolism of Aromatic Compounds and Trichloroethylene†

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*Pseudomonas cepacia* G4 possesses a novel pathway of toluene catabolism that is shown to be responsible for the degradation of trichloroethylene (TCE). This pathway involves conversion of toluene via o-cresol to 3-methylcatechol. In order to determine the enzyme of toluene degradation that is responsible for TCE degradation, chemically induced mutants, blocked in the toluene ortho-monoxygenase (TOM) pathway of G4, were examined. Mutants of the phenotypic class designated TOM A− were all defective in their ability to oxidize toluene, o-cresol, m-cresol, and phenol, suggesting that a single enzyme is responsible for conversion of these compounds to their hydroxylated products (3-methylcatechol from toluene, o-cresol, and m-cresol and catechol from phenol) in the wild type. Mutants of this class did not degrade TCE. Two other mutant classes which were blocked in toluene catabolism, TOM B+, which lacked catechol-2,3-dioxygenase, and TOM C−, which lacked 2-hydroxy-6-oxoheptadienoic acid hydrolase activity, were fully capable of TCE degradation. Therefore, TCE degradation is directly associated with the monooxygenation capability responsible for toluene, cresol, and phenol hydroxylations.

Trichloroethylene (TCE) is the most frequently encountered priority pollutant of groundwaters of the United States (26). TCE remains a persistent compound in the environment despite demonstrations of its degradation by several bacteria. For example, *Pseudomonas putida* F1 (22, 30), *Pseudomonas mendocina* (32), *Pseudomonas cepacia* G4 (18, 20–22), *Pseudomonas pickettii* PKO1 (15), two methanotrophic bacteria, *Methylosinus trichosporium* OB3b (24, 28) and an unidentified methanotrophic isolate, 46-1 (17), the lithotrophic *Nitrosomonas europaea* (1), several propane-utilizing *Mycobacterium* isolates (29), isoprene-utilizing *Alcaligenes denitrificans* subsp. *xylsioxidans* JE75, and *Rhodococcus erythropolis* JE77 (9) have all been reported to degrade TCE.

Methanotrophs degrade TCE via a soluble methane monooxygenase (11). *Nitrosomonas europaea* is presumed to use ammonia monooxygenase, and *A. denitrificans* and *R. erythropolis* are thought to utilize propene monooxygenases. TCE degradation by *P. putida* F1 has been shown to be catalyzed by toluene dioxygenase (20, 29). However, methane monooxygenase and toluene dioxygenase actions on TCE result in an autoactivation of the enzymes by a mechanism that leads to covalent modification of cellular components (resulting in toxicity to *P. putida* F1) (11, 31). The para-acting toluene monooxygenase of *P. mendocina* is responsible for degradation of TCE by this organism (32), as is the meta-acting toluene monooxygenase of *P. pickettii* PKO1 (15). *P. cepacia* G4 degrades TCE when induced by a variety of aromatic compounds including toluene, phenol, o-cresol, m-cresol, and benzene (20, 22). A novel pathway of toluene degradation by *P. cepacia* G4 has been described in which toluene is sequentially hydroxylated to form first o-cresol and then 3-methylcatechol (27). Phenol-induced cultures of *P. cepacia* G4 efficiently degrade TCE at concentrations up to 180 μM (10). To determine the enzyme(s) responsible for TCE degradation, mutants of *P. cepacia* G4 that were defective in toluene catabolism were isolated. Analyses of these mutants suggest that an enzyme(s) of *P. cepacia* G4 catalyzes monooxygenation of toluene at the ortho position and may be responsible for TCE degradation. Evidence is presented to suggest that this enzyme may also catalyze the conversion of o-cresol to 3-methylcatechol.

**MATERIALS AND METHODS**

*Bacterial strains and chemicals.* The isolation, characterization, and identification of *P. cepacia* G4 have been previously described (18, 21). Carbenicillin, d-cycloserine, piperacillin, formamide, N-methyl-N-nitro-N-nitrosoguanidine (NTG), triphenylenetrazolium chloride (TTC), and m-trifluoromethylphenol (TFMP) were obtained from Sigma Chemical Co., St. Louis, Mo. A culture of *P. putida* U (ATCC 17514) was obtained from the collection of the late S. Dagley, University of Minnesota (2, 6).

*Culture conditions.* Basal salts medium (BSM) (13) containing 20 mM sodium lactate was used for the routine growth of G4. Solid media containing BSM were prepared by incorporation of purified agar (Difco) to 1.6%. Phenol at 2 mM and o-cresol, m-cresol, and catechol at 1 mM were supplied as the sole carbon sources by addition of filter-sterilized solutions to autoclaved BSM. *P. putida* U was grown in BSM containing 5 mM succinate and 2 mM phenol. Unless otherwise noted, all incubations were carried out at 30°C with rotary shaking.

Growth of G4 on toluene was accomplished by bubbling a sterile air-toluene mixture into a 125-ml Erlenmeyer flask fitted to a Sherer glass impinger with a 24/40 ground glass joint (Ace Glass Inc., Vineland, N.J.) which contained 50 ml of BSM and the cell inoculum. This sterile air-toluene mixture was generated by passing an air stream (at 200 to 250 ml min−1) into a 1-liter Erlenmeyer flask containing 10 ml of
toluene and out through a 0.2-μm-pore-size membrane filter (Nucleopore). G4 was grown with toluene as the sole carbon source on solid media by directing air-entrained toluene into a 40-liter glass chamber (inverted aquarium) (25) by 35 by 46 cm) in which carbon-free plates were stacked at delivery rates of 0.3 to 0.7 liters min⁻¹. Distinct colonies were evident within 3 days.

Mutagenesis. An overnight culture of G4 grown on BSM-lactate was diluted in fresh medium (12.5% inoculum) containing NTG (50 μg mL⁻¹). At 30, 45, 60, 90, and 120 min, 5-ml subsamples were removed, washed twice with 5 ml of BSM, suspended in 5 ml of BSM-lactate, and held overnight at 0°C. Samples exhibiting between 70 and 90% cell death (determined by dilution plating the cells to lactate plates and then comparing them with untreated cells) were pooled. Mutants unable to use toluene were enriched by the antibiotic counterselection method of Orston et al. (25) with toluene vapor as the sole growth substrate. The counterselective antibiotics piperacillin (7) and D-cycloserine were employed at 140 and 200 μg mL⁻¹, respectively. Counterselection involved three cycles of growth with toluene in the presence of these antibiotics alternated with growth on lactate (20 mM) in the absence of antibiotics.

Mutant detection. Survivors of mutagenesis and counterselection were screened for possible toluene catabolic mutations by using either TTC or TFMP as the indicator of toluene catabolic activity.

The survivors were diluted, and half were spread to 40 indicator plates (containing purified agar [1.6%], TTC [0.025 mg mL⁻¹], and peptone [0.1 mg mL⁻¹] in BSM) and grown in the presence of toluene in the vapor chamber. Colony selection was based on the TTC dye reduction principle of Bochner and Savageau (4), in which colorless TTC is reduced to red triphenyl tetrazolium chloride-formazan in the presence of excess cellular reductants. Wild-type G4 formed colorless colonies in the presence of TTC without an additional carbon source; in the presence of toluene vapor they became dark red. Mutant colonies unable to utilize toluene failed to reduce TTC and therefore remained colorless in the presence of toluene vapor. Putative mutants identified in this manner were then tested for substrate utilization, colored metabolite formation, and transformation of TFMP (see below).

The remaining half of the counterselection survivors were plated on BSM-lactate plates. Single colonies were picked to duplicate BSM-lactate plates (maximum, 50 per plate). These isolates were tested for their ability to transform TFMP. G4 can metabolize TFMP to a yellow product (7,7,7-trifluorour-2-hydroxy-6-oxo-2,4-heptadienoic acid [TFHA]) (8). Wild-type G4 colonies grown overnight on BSM-lactate plates in the presence of toluene vapor turned yellow within 30 min when sprayed with 5 mM TFMP.

Oxidation of aromatic compounds. Oxygen uptake rates for toluene, phenol, o-cresol, or 3-methylcatechol (all at 1 mM) were determined by using whole cells grown overnight with 20 mM lactate and 2 mM phenol and induced the next morning with phenol (2 mM) for an additional 2 h. Cells were washed with and suspended in BSM to an A₅₀₀ of 2.0. Measurements of oxygen uptake were performed with a Gilson oxygraph equipped with a Clark electrode (Gilson Medical Electronics, Inc., Middleton, Wis.) in a total reaction volume of 2 ml (consisting of 0.05 ml of cells, 1.93 ml of BSM, and 0.02 ml of 0.1 M carbon source) as previously described (20).

Enzyme assay conditions. Overnight cultures grown in BSM-lactate medium were induced by toluene-vapor exposure during the final 6 h of incubation. Cells were harvested by centrifugation, washed in 100 ml of BSM, and stored as a pellet at −70°C. Cell extracts were prepared by the method of Hughes (14) as previously described (27). Catechol-2,3-dioxygenase activity was measured by the procedure of Nozaki et al. (23) as described by Gibson (12). Hydroxymuconic semialdehyde hydrolase activity was determined by the rate of decrease in 2-hydroxy-6-oxohepta-2,4-dienoate (molar extinction coefficient at 388 nm ε₂₈₈ = 13,800) or 2-hydroxymuconic semialdehyde (ε₂₇₅ = 33,000) concentrations. Solutions of each substrate were produced by using heat-treated cell extracts of phenol-grown P. putida U as described by Dagley and Gibson (6). 2-Hydroxymuconic semialdehyde hydrolase activity was determined as described by Bayly and Wigmore (3) in 3-ml reaction mixtures containing 145 μmol of potassium phosphate buffer (pH 7.5), 1 μmol of catechol or 3-methylcatechol, and heat-treated P. putida U cell extract (20 to 50 μg of protein). After complete conversion of catechol to 2-hydroxymuconic semialdehyde or of 3-methylcatechol to 2-hydroxy-6-oxohepta-2,4-dienoate by the P. putida U enzyme, cell extracts of G4 or the mutant strains were added and the rate of 2-hydroxymuconic semialdehyde or 2-hydroxy-6-oxohepta-2,4-dienoate disappearance was determined.

The increase in the rate of 2-hydroxymuconic semialdehyde disappearance after addition of 0.3 μmol of NAD⁺ (corrected for activity due to the P. putida U extract alone) was reported as hydroxymuconic semialdehyde dehydrogenase activity. Production of cis-cis-muconate from catechol by catechol-1,2-dioxygenase was monitored at 260 nm (cis-cis-muconate ε₂₆₀ = 16,900) (12). Inactivation of catechol-2,3-dioxygenase activity with 30 mM H₂O₂ was performed as described by Murray and Williams (19). Protein concentration was determined by the method of Bradford (5) with bovine serum albumin as a standard.

Chemical analyses. TCE concentrations were determined by gas chromatographic analysis of pentane extracts of the reaction vessel and its contents (20) and reported as though all the remaining TCE was in aqueous solution. High-pressure liquid chromatography (HPLC) analyses were performed as previously described (27).

RESULTS

Metabolism of TFMP. A culture of phenol-induced G4, exposed to 5 mM TFMP, became yellow within 5 min. After 30 min, cells were removed by centrifugation and the UV-visible spectrum of the supernatant was recorded. The absorbance spectrum of the yellow supernatant (data not shown) closely matched that of the presumed ring cleavage product 7,7,7-trifluoro-2-hydroxy-6-oxo-2,4-heptadienoic acid (THFA) (absorbance maximum at pH 7.4, 385 nm; ε₂₈₅ = 26,900) reported by Engesser et al. (8). On the basis of this data and the previously demonstrated ability of G4 to produce o-cresol from toluene (27), a pathway is proposed for THFA formation by G4 (Fig. 1).

Mutants defective in toluene catabolism. Two procedures were used for preliminary selection of mutants defective in toluene catabolism following NTG mutagenesis and antibiotic counterselection. Both the TFMP color reaction and the TTC dye reduction assays revealed colonies that were unable to effect the color changes seen with wild-type cells. In addition, certain colonies were identified, in both procedures, which were able to form a diffusible brown product in the presence of toluene or phenol. These colonies were presumed to lack active catechol-2,3-dioxygenase, causing
the accumulation of catechol which autoxidizes to soluble brown products in the test media. Production of this brown color obscures the assessment of color reactions with TFMP and TTC. Conversely, colonies failing to produce either the brown color or the yellow product from TFMP were assumed to be defective in reactions preceding catechol ring cleavage.

Each putative mutant was tested for its ability to grow with tolune, phenol, or o- or m-cresol and for the production of yellow or brown products when grown on BSM-lactate in the presence of phenol or tolune. From 449 putative mutants identified (189 by the TFMP procedure and 260 by the TTC screen), three distinct mutant classes could be discerned (Table 1). The first class, of which G4 100 is typical, failed to utilize any aromatic substrates or to form any colored products. The second class, typified by G4 102, produced a brown discoloration of the medium in the presence of all aromatic substrates and grew with only phenol but at rates considerably slower than those of the wild-type cells (as judged by the fact that the colonies were much smaller than those of the wild type after 3 days of growth). Mutant G4 103 was typical of the third class and formed a yellow coloration in the presence of all tested aromatics except for phenol. The UV-visible spectrum of this yellow supernatant (data not shown) matched that of 2-hydroxy-6-oxoheptadionic acid (2). This third class retained the ability to grow with phenol as the sole carbon source. After induction by growth on lactate in the presence of phenol, cells of G4 103 formed a yellow color from TFMP.

G4 100 and nine other mutants with indistinguishable phenotypes were tested for reversion by examining for colonies with the ability to grow with tolune vapor as the sole carbon source. G4 100 and two of the others gave rise to spontaneous revertant tolune-utilizing colonies. No revertants were detected for the remaining seven. Revertants of G4 100 appeared at a frequency of $1 \times 10^{-7}$ (with the other two reverting at frequencies of $1.6 \times 10^{-8}$ and $3 \times 10^{-9}$). One such revertant of G4 100 (designated strain G4 100R) was purified and was shown to have reacquired the ability to produce TFHA from TFMP and to utilize all the substrates supporting growth of wild-type G4.

**Oxygen uptake and enzyme activities.** Phenol-induced cells were screened for their abilities to oxidize tolune, phenol, o-cresol, and 3-methylcatechol. G4 100 and 16 other phenotypically indistinguishable isolates did not exhibit significant oxygen uptake activity with tolune but did utilize $\approx 330$ nmol of $O_2 \text{ min}^{-1}$ with 3-methylcatechol. Four of these isolates (including G4 100) were tested and showed no O$_2$ uptake with phenol or o-cresol. HPLC analysis of the culture supernatant of G4 100 failed to indicate disappearance or transformation of tolune or phenol. G4 100R exhibited rates of oxygen consumption similar to those of wild-type cells with o-cresol (230 nmol of oxygen min$^{-1}$ mg of protein$^{-1}$) and reduced rates with tolune and phenol (40 and 163 nmol of oxygen min$^{-1}$ mg of protein$^{-1}$, respectively). G4 102, which produced a brown discoloration of the medium in the presence of phenol or tolune, did not demonstrate significant oxygen uptake rates with tolune, phenol, or o-cresol, as G4 100 did, but it was distinguishable by its failure to oxidize catechol or 3-methylcatechol. G4 103 exhibited rates of oxidation of tolune, phenol, and o-cresol that were only 20 to 30% that of wild-type cells.

**Enzyme activities were determined for representative tolune-induced cultures of each mutant class (Table 2).** Cell extracts of G4 100 demonstrated activities similar to those of the wild type for all enzymes assayed. This agrees with the

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**TABLE 1. Mutant phenotypes**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotypic class</th>
<th>Action on TFMP induced with:</th>
<th>Growth on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tolune</td>
<td>Phenol</td>
</tr>
<tr>
<td>G4</td>
<td>Wild type</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G4 100</td>
<td>TOM A$^-$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G4 100R</td>
<td>TOM A$^-$ revertant</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G4 102</td>
<td>TOM B</td>
<td>Br</td>
<td>Br</td>
</tr>
<tr>
<td>G4 103</td>
<td>TOM C$^-$</td>
<td>Ye</td>
<td>+</td>
</tr>
</tbody>
</table>

$^a$ Conversion of TFMP to its yellow ring cleavage product is indicated by "+.", Failure to do so or failure to grow on indicated carbon sources is indicated by "-.", $^b$ Br and Ye, TFMP conversion was not detectable in these colonies because of accumulation of brown (Br) or yellow (Ye) metabolites in the presence of this inducer.

$^b$ Very slow growth (5 to 7 days for pinpoint colonies).

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**TABLE 2. Toluene-induced enzyme activities**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Catechol-2,3-dioxygenase</th>
<th>Hydrolase</th>
<th>Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cat</td>
<td>3mCat</td>
<td>Hms</td>
</tr>
<tr>
<td>G4</td>
<td>6,550</td>
<td>3,780</td>
<td>112</td>
</tr>
<tr>
<td>G4 100</td>
<td>5,290</td>
<td>1,970</td>
<td>195</td>
</tr>
<tr>
<td>G4 100R</td>
<td>3,390</td>
<td>5,900</td>
<td>281</td>
</tr>
<tr>
<td>G4 102</td>
<td>0.3</td>
<td>0.6</td>
<td>148</td>
</tr>
<tr>
<td>G4 103</td>
<td>1,680</td>
<td>1,210</td>
<td>0.2</td>
</tr>
</tbody>
</table>

$^a$ Enzyme activities of cell extracts of tolune-induced cells. Values are the average of duplicate determinations following induction with tolune. Substrate abbreviations: Cat, catechol; 3mCat, 3-methylcatechol; Hms, 2-hydroxymuconic semialdehyde; Hod, 2-hydroxy-6-ketohexa-2,4-dienoate.
conclusions made from oxygen uptake studies and suggests that enzymes metabolizing catechol or 3-methylcatechol to 2-ketopent-4-enoate and beyond were present and inducible. Hydrolase and dehydrogenase activities toward 2-hydroxymuconic semialdehyde appear to be slightly elevated relative to wild-type levels in G4 100. Extracts of G4 103 did not possess detectable hydrolase activity toward either 2-hydroxymuconic semialdehyde or its methyl homolog 2-hydroxy-6-oxohepta-2,4-dienoic acid. The absence of any yellow metabolite accumulation in the presence of phenol by cells of G4 103 is explained by the demonstrated activity of hydroxy-

\[
\text{CH}_3
\]

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenol (μM) remaining</th>
<th>Toluene (μM) remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>G4</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>G4 100</td>
<td>3.67 ± 0.03</td>
<td>3.56 ± 0.46</td>
</tr>
<tr>
<td>G4 100R</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>G4 102</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

* Means of triplicate TCE determinations (± standard deviation) using 24-h lactate cultures induced by exposure to toluene vapor or 2 mM phenol for 2 h. The amount of TCE remaining in uninoculated cultures was 3.72 ± 0.07 μM.

Catechol-2,3-dioxygenase activity was presumed to be absent in G4 102 because this strain accumulated presumed catechol breakdown products when incubated with phenol or toluene. This was confirmed by enzyme assays (Table 2). G4 102 also exhibited a loss of ability to produce a small increase in A260 following the addition of catechol (data not shown). This was initially interpreted as a loss of catechol-1,2-dioxygenase activity. However, this apparent catechol-1,2-dioxygenase activity in toluene-induced G4 cell extracts was lost after treatment with 30 mM H2O2 (data not shown). Loss of this activity after exposure to H2O2 (a treatment shown to inactivate catechol-2,3-dioxygenase but not catechol-1,2-dioxygenase in P. putida extracts [19]) suggested that the small change in A260 (the absorption maximum of cis-cis-muconate) was probably caused by the formation of 2-hydroxymuconic semialdehyde (absorption maximum, 375 nm) through the action of catechol-2,3-dioxygenase on phenol.

**TCE degradation.** The ability of representatives of each mutant class to degrade TCE after induction with either

FIG. 2. Summary of the observed catabolic transformation of toluene, o-cresol, m-cresol, and phenol. For each step a mutant phenotypic class is given.
phenol (2 mM) or toluene (vapor) was determined. G4 100 was the only mutant lacking the ability to degrade TCE (Table 3). This mutant also failed to initiate attack on toluene, o-cresol, m-cresol, and phenol (HPLC analyses not shown). One other TOM A− strain was also tested for TCE degradation with identical results (data not shown). This mutant was apparently not a sibling, since (unlike G4 100) it did not spontaneously revert to phenol and toluene utilization. Revertants of this mutant were found only after reexposure to NTG.

**DISCUSSION**

Mutants of *P. cepacia* G4 unable to catabolize toluene were organized into three phenotypic classes (TOM A−, TOM B−, and TOM C−) according to patterns of hydrocarbon utilization, the results of chromogenic reactions, and enzyme assays (Fig. 2). Though only one representative of each class is reported in detail, there were 110 of the TOM A− type, 95 of the TOM B− type, and 41 of the TOM C− type initially screened from a total of 449 putative NTG-generated mutants selected by TFMP and TTC indicator methods.

Mutants belonging to the TOM A− class (e.g., G4 100) possess no detectable activity towards toluene, o-cresol, m-cresol, or phenol. We suggest that this phenotype is caused by a mutation either in a structural gene encoding the oxygenase previously shown to be responsible for hydroxylation of the aromatic ring of toluene and/or the cresols (27) or in a regulatory component that prevents synthesis of this enzyme(s). This class was shown to retain the enzymes requisite for the oxidation of catechol or 3-methylcatechol and metabolism of their ring fission products. These findings, together with the observed frequency of reversion to a wild phenotype (10−6 to 10−5), are consistent with the hypothesis that a single genetic lesion is responsible for this phenotype. In this study the only mutants of G4 that are impaired for TCE degradation are those belonging to the TOM A− class.

Therefore, the enzyme(s) required for metabolism of TCE is that required for monooxidation of toluene, phenol, o-cresol, and m-cresol. It is possible that representatives of the TOM A− class have polar mutations that impair the expression of two or more genes, one whose product is responsible for the hydroxylation of toluene and another whose product is responsible for the hydroxylation of phenol and the cresols.

The mutant class TOM B− (e.g., G4 102) is defective for catechol-2,3-dioxigenase activity. Mutants of this class were readily identified by their growth and oxygen uptake patterns and the accumulated products expected for a mutant lacking this enzyme. Indeed, strain G4 102 has previously been shown to accumulate 3-methylcatechol from toluene (27).

In an earlier study (20) wild-type G4 was reported to possess toluene-inducible catechol-1,2-dioxigenase activity. The absence of any detectable level of this enzyme in G4 102 (where no catechol-2,3-dioxigenase activity can be shown) suggests that small changes in the A260 previously attributed to catechol-1,2-dioxigenase activity are instead caused by rapid formation of 2-hydroxymuconic semialdehyde. The presence of catechol-1,2-dioxigenase activity in G4 under phenol-induced conditions appears likely since TOM B− strains are capable of slow growth on phenol. During growth with toluene or cresols, the third mutant class described, TOM C− (e.g., G4 103), was shown to accumulate a yellow metabolite with the UV-visible spectrum of 2-hydroxy-6-oxoheptadienoic acid. Mutants of this class lacked the 2-hydroxy-6-oxohepta-2,4-dieneoic hydrolase activity which is necessary for toluene degradation but which is not required for phenol catabolism. While cell extracts of TOM C− strains could hydrolyze 2-hydroxymuconic semialdehyde (the corresponding phenol metabolite), its principal route of metabolism appeared to occur via NAD-dependent dehydrogenation of 2-hydroxymuconic semialdehyde. Extracts of G4 were shown to have hydrolase activity that was 12-fold greater towards 2-hydroxy-6-oxohepta-2,4-dieneoic than...
towards 2-hydroxymerconic semialdehyde (Table 2). Since the NAD-dependent 2-hydroxymerconic semialdehyde dehydrogenase does not act on 2-hydroxy-6-oxohepta-2,4-dienoate, its accumulation in the presence of toluene and the absence of any accumulation of 2-hydroxymerconic semialdehyde in the presence of phenol by the TOM C– strain G4 103 are explained.

The description of another toluene catabolic pathway that includes an enzyme with activity towards TCE now brings the total number of such known pathways to four: the toluene dioxygenation pathway of P. putida F1 (22, 30), the toluene o-monoxygenation pathway of G4 (27), the toluene m-monoxygenation pathway of P. pickettii PKO1 (15, 16), and the toluene p-monoxygenation pathway of P. mendocina (32) (Fig. 3). Another toluene-degradative pathway that is initiated by the toluene monoxygenase of the TOL plasmid (pWWO) (33) involves an attack upon the methyl group rather than the aromatic ring of toluene. Enzymes of this pathway fail to act on TCE (22). TCE is degraded in all cases in which an oxygenase that directs the addition of molecular oxygen to the aromatic nucleus of toluene is expressed. This would indicate that common features are shared between TCE and the aromatic ring of toluene which allow the toluene ring oxygenases to accept both as substrates.

In view of the designation of the toluene dioxygenase genes of P. putida F1 as tod (34) and the designation of para-toluene dioxygenase of P. mendocina as TMO (32), we propose the gene designation tom for the genes of the P. cepacia toluene ortho-monoxygenase pathway.

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Catabolic mutants of *P. cepacia* G4


