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Combination of the *tod* and the *tol* Pathways in Redesigning a Metabolic Route of *Pseudomonas putida* for the Mineralization of a Benzene, Toluene, and *p*-Xylene Mixture

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Construction of a hybrid strain which is capable of mineralizing components of a benzene, toluene, and *p*-xylene mixture simultaneously was attempted by redesigning the metabolic pathway of *Pseudomonas putida*. Genetic and biochemical analyses of the *tod* and the *tol* pathways revealed that dihydrodiols formed from benzene, toluene, and *p*-xylene by toluene dioxygenase in the *tod* pathway could be channeled into the *tol* pathway by the action of *cis*-*p*-toluate-dihydrodiol dehydrogenase, leading to complete mineralization of a benzene, toluene, and *p*-xylene mixture. Consequently, a hybrid strain was constructed by cloning *todC1C2BA* genes encoding toluene dioxygenase on RSF1010 and introducing the resulting plasmid into *P. putida* mt-2. The hybrid strain of *P. putida* TB105 was found to mineralize a benzene, toluene, and *p*-xylene mixture without accumulation of any metabolic intermediate.

In recent decades, aromatic compounds used as agricultural or industrial chemicals have become an integral part of our economy. They are deliberately or accidentally released into the environment in large amounts and accumulate in soil and surface water, leading to serious damage to our living conditions. One particular concern is the contamination of drinking-water sources by benzene, toluene, and *p*-xylene (BTX), which are typical petroleum components.

Microbial degradation of these compounds in aquatic environments can serve as a significant remediation mechanism (4, 24). The individual compounds constituting BTX mixtures are reported to be easily degraded by the microorganisms of soil and surface water. Catabolic degradation of BTX via two typical pathways (the *tod* and the *tol* pathways) has been extensively studied (8–12, 25–27). However, most of the laboratory investigations concerning the biodegradation of BTX have focused on one of the individual compounds, neglecting the fact that environmental contamination by individual components of BTX mixtures is uncommon in real situations.

Although each individual BTX compound can be mineralized by natural microorganisms, the three components of BTX mixtures cannot be successfully mineralized simultaneously. Biochemical studies of the *tod* and the *tol* pathways have revealed that this incomplete biodegradation of the BTX mixture is due to the fact that 3,6-dimethylcatechol formed from *p*-xylene is a dead-end product in the *tod* pathway (10). Furthermore, xylene oxygenase in the *tol* pathway cannot recognize benzene as a substrate (25). Even when a microbial consortium is applied, substrate interactions may negatively affect the complete biodegradation of BTX mixtures (1, 15, 19).

Although microorganisms in soil and water have considerable evolutionary potential, the evolution of effective pathways for certain compounds may be rather slow. Recently introduced genetic engineering techniques offer considerable promise as an approach to accelerating the evolution of desired metabolic pathways. Two general strategies can be envisaged for the experimental evolution of new catabolic activities: the restructuring of an existing pathway and the assembly of a new hybrid route by the combination of appropriate sections of different pathways. These strategies have been successfully applied to the construction of novel pathways through which recalcitrant haloaromatics and alkylaromatics can be degraded (20, 22).

In this work, we attempted to construct a hybrid pathway in a *Pseudomonas putida* strain through which components of a BTX mixture can be simultaneously mineralized. For this purpose, biochemical and genetic analyses of the enzymes at each catabolic step in the *tod* and the *tol* pathways were investigated and the critical step connecting the two pathways was determined.

MATERIALS AND METHODS

Chemicals. Benzene, toluene, *p*-xylene, catechol, and 4-methylcatechol were obtained from Sigma (St. Louis, Mo.). *cis*-Benzene-dihydrodiol (BCG) and *cis*-toluene-dihydrodiol (TCG) were obtained from A. J. Blacker (Zeneka Bio Products, Billingham, United Kingdom). *cis*-*p*-Xylene-dihydrodiol (XCG), 3,6-dimethylcatechol, 3-methylcatechol, and *cis*-*p*-toluate-dihydrodiol (TACG) were biologically prepared as described previously (10, 13, 26). All other chemicals were of reagent grade.

Bacterial strains and plasmids. Microorganisms and plasmids used in this study are listed in Table 1. *Pseudomonas putida* F1 was a gift from D. T. Gibson (University of Iowa, Iowa City), and pPL392 was obtained from S. Harayama (Marine Biotechnology Center, Kamaishi City, Japan).

Media and culture conditions. *P. putida* strains were grown in Luria-Bertani (LB) media at 30°C, and *Escherichia coli* strains were grown at 37°C. Ampicillin or streptomycin, when needed to select for the presence of plasmids, was added at a concentration of 100 or 200 μg/ml, respectively. In order to isolate the transformants exhibiting the toluene dioxygenase activity, 1 mM indole was also added to the LB agar plates.

Construction of pTOD037 carrying *todC1C2BA* genes encoding toluene dioxygenase. The entire base sequence of the *todC1C2BADE* genes was previously reported by Zylstra and Gibson (26). On the basis of this base sequence, the genomic DNA of *P. putida* F1 was cut with *Eco*RI and *Xho*I to obtain the DNA fragment containing *todC1C2BADE* genes, which encode the first three enzymes (toluene dioxygenase, TCG dehydrogenase, and catechol-2,3-dioxygenase) in the

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Characteristics	Reference(s) or source
Microorganisms		
P. putida mt-2 (ATCC 23793)	Contains TOL plasmid pWW0	American Type Culture Collection
P. putida F1	Contains *tod* operon on chromosome	26, 27
P. putida (ATCC 39119)	Produces TACG from *p*-xylene	13
E. coli C600	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*	Pharmacia (Piscataway, N.J.)
E. coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ΔM15]	Pharmacia
Plasmids		
RSF1010	Broad-host-range plasmid (Sm^r Su^r)	23
pPL392	pBR322 containing *meta* operon of TOL plasmid pWW0	11
pKK223-3	Contains *tac* promoter	Pharmacia
pTOD037	RSF1010 containing *todC1C2BA* genes of *tod* operon of *P. putida* F1	This work
pTOL047	pKK223-3 containing *xylL* of TOL plasmid pWW0	This work

pathway for catabolism of toluene by *P. putida* F1. The resulting 6- to 7-kb DNA fragment was obtained by agarose gel electrophoresis. The DNA fragment was cloned in pBlueScriptSK+, resulting in the hybrid plasmid pTOD001, and this plasmid then was introduced into *E. coli* JM109. The transformants possessing *todC1C2BADE* were isolated on LB agar plates containing 1 mM indole. Colonies in which the blue pigment indigo is formed by the action of toluene dioxygenase were selected (7). From the selected transformant, pTOD001 was isolated and digested with *Nco*I. A single *Nco*I site is located in the middle of the *todD* gene, which specifies the TCG dehydrogenase. *Eco*RI linker was attached to the *Nco*I-digested pTOD001, and the plasmid was ligated. Thus, by digesting pTOD001 with *Eco*RI, we obtained a DNA fragment containing only *todC1C2BA* genes, which encode the toluene dioxygenase enzyme system composed of reductase$_{TOL}$ (*todA*), ferredoxin$_{TOL}$ (*todB*), and two subunits of the terminal dioxygenase (*todC1C2*). This DNA fragment was cloned in RSF1010 digested with *Eco*RI, leading to the formation of the plasmid pTOD037. Transformation of *E. coli* JM109 with pTOD037 and spreading of these transformants on LB agar plates containing indole and streptomycin gave blue-pigment-forming colonies. Each transformant producing blue pigment was cultivated in LB media with benzene, and culture broth was analyzed by high-performance liquid chromatography (HPLC) to examine the ability to transform benzene to BCG. Formation of BCG from benzene confirmed the expression of *todC1C2BA* genes, which encode the toluene dioxygenase.

Construction of pTOL047 carrying a *xylL* gene encoding TACG dehydrogenase. The cloning of a *xylL* gene encoding TACG dehydrogenase was conducted by PCR based on the base sequence of the *meta*-cleavage genes of the TOL plasmid pWW0 reported by Neidle et al. (18). The sequences of the primers used were 5′-CGCGAATTCCATGAACAAGCGTTTCCAGCGC-3′ and 5′-CCACTGCAGACATAAGGGTCAGCCG-3′. The underlined sequences at the 5′ end of each oligonucleotide indicate restriction sites (*Eco*RI and *Pst*I, respectively). pPL392 was used as a template. DNA amplification was achieved by a 30-cycle PCR protocol with a 30-s denaturing step at 94°C, a 30-s annealing step at 55°C, and a 20-s extension step at 72°C. The amplified DNA was precipitated with ethanol, digested with *Eco*RI and *Pst*I, and separated on a 1% agarose gel. The purified fragment was ligated into the expression vector pKK223-3, which had been cleaved with *Eco*RI and *Pst*I. The resulting plasmid was designated pTOL047. pTOL047 was transformed into *E. coli* JM109 with selection on ampicillin-containing LB agar.

DNA manipulation. All DNA manipulations performed in this study were done as described by Brown (3).

Studies of the activities of the enzymes of the *tol* pathway. A crude extract of *E. coli* C600(pPL392) or *E. coli* JM109(pTOL047) was used for the studies of the enzymes of the *tol* pathway involved in the degradation of dihydrodiol compounds. Each of the strains was grown in LB medium supplemented with ampicillin. Benzoate at 1 mM as an inducer of the P_M promoter of pPL392 was added to the culture of *E. coli* C600(pPL392) when the turbidity of the culture reached a value of 0.5, and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture of *E. coli* JM109(pTOL047) for induction of the *tac* promoter of pTOL047 when the turbidity reached a value of 0.7. Cells were harvested by centrifugation at 12,000 × *g* for 10 min at 4°C and resuspended in the mineral salt medium. Crude extract was prepared by ultrasonic treatment of cell suspension for 3 min by using a sonicator (Branson Sonic Power Co., Danbury, Conn.). The concentration of protein in the crude extract was measured by the Bradford method (2).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) slab gels of whole-cell extract were prepared by the method of Laemmli (14). Gels (0.75 mm thick) were run at 15 mA for the stacking gel (4%) and at 24 mA for the separating gel (12%) until the tracking dye reached the bottom of the gel. Proteins were visualized by staining with Coomassie blue G250.

Biodegradation of BTX. Unless otherwise stated, analysis of BTX biodegradation was performed by using 100-ml serum vials containing 5 ml of mineral salt medium at 30°C with gentle shaking. The serum vial was tightly sealed with a Teflon-coated rubber stopper to prevent the loss of BTX by evaporation. Cells were initially grown in LB medium without any BTX compounds for about 10 to 12 h, harvested by centrifugation at 12,000 × *g* for 10 min (4°C), and resuspended in the mineral salt medium for biodegradation experiments. The initial cell concentration in the vial was about 0.4 mg (dry weight) per ml. BTX compounds were injected as pure stock with a 10-μl syringe. The mineral medium contained the following: 5.8 g of KH_2PO_4, 4.5 g of K_2HPO_4, 2.0 g of $(NH_4)_2SO_4$, 0.34 g of $MgCl_2 \cdot 6H_2O$, 0.02 g of $CaCl_2$, 0.002 g of $FeSO_4 \cdot 7H_2O$, and 0.0016 g of $MnCl_2 \cdot 4H_2O$ per liter of distilled water.

Analytical methods. The concentrations of BTX compounds were determined by gas chromatography as described in our previous report (5). BCG, TCG, XCG, catechol, 4-methylcatechol, and 3,6-dimethylcatechol were analyzed by using an HPLC (model LC9A; Shimadzu, Kyoto, Japan) equipped with a UV-visible light (UV/VIS) detector (model SPD6AV; Shimadzu). An octyldecyl silane column [CLC-ODS(M), 15 cm long] (YMC, Wilmington, N.C.) was used with a methanol-water-phosphoric acid mixture (370:630:1 [vol/vol/vol]) as a mobile phase. The flow rate of eluent was 1.5 ml/min. All compounds listed above were detected at 254 nm. The detection limit for HPLC analysis was around 0.045 mM for catechol. HPLC-mass spectrometry (LC/MS) analyses were carried out by using an HPLC (HP 1050 series; Hewlett-Packard, Avondale, Pa.) coupled with a VG Quattrotriple quadrupole MS equipped with an atmospheric pressure chemical ionization interface (Fisons Instrument/VG Biotech, Altrincham, United Kingdom). The atmospheric pressure chemical ionization interface was operated in a negative-ion mode with a corona discharge at 3.0 kV. This LC/MS system produces a negatively charged molecular ion with a mass 1 unit less than the molecular weight of the analyte by hydrogen abstraction. HPLC separation was conducted by the procedure described above except that the flow rate of eluent was fixed at 0.35 ml/min. The conversion of NAD^+ to NADH was monitored by using a UV/VIS spectrophotometer (model UV2100; Shimadzu) at 340 nm (21).

RESULTS AND DISCUSSION

Redirection of the metabolic flux of BTX from the *tod* pathway to the *tol* pathway. Two metabolic barriers which prevent the complete mineralization of BTX mixtures have been known to be imposed on the *tod* and the *tol* pathways: 3,6-dimethylcatechol produced from *p*-xylene cannot be metabolized further in the *tod* pathway, and benzene cannot be assimilated in the *tol* pathway. If the *tol* pathway could mineralize either 3,6-dimethylcatechol or dihydrodiol compounds, both of which are initial metabolic intermediates in the *tod* pathway, it might be possible to bypass the metabolic barriers by hybridizing the *tod* and the *tol* pathways.

When *P. putida* mt-2 was incubated with 3,6-dimethylcatechol as the sole carbon and energy source for 48 h, no decrease in the concentration of 3,6-dimethylcatechol was observed. This implies that the mineralization of BTX mixtures cannot be achieved by the hybridization of the intact *tod* and the *tol* pathways because 3,6-dimethylcatechol produced by the partial oxidation of *p*-xylene in the *tod* pathway will remain unattacked. We also performed a mixed cultivation of *P. putida*

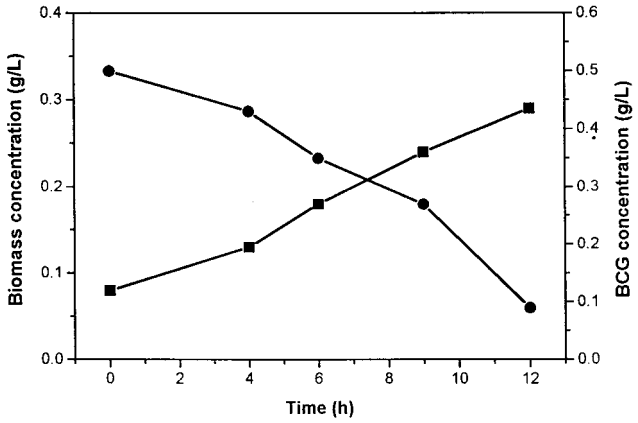

FIG. 1. Typical profile of biodegradation of BCG by *P. putida* mt-2. Symbols: ■, biomass; ●, BCG.

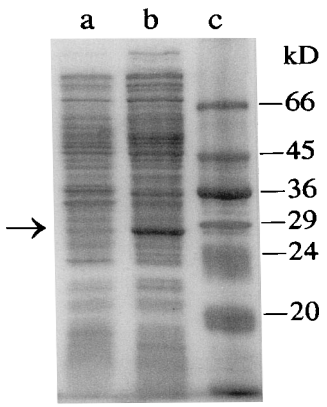

FIG. 2. SDS-PAGE of crude extracts showing overexpression of TACG dehydrogenase in *E. coli* JM109(pTOL047). Lanes: a, *E. coli* JM109(pKK223-3); b, *E. coli* JM109(pTOL047); c, standard proteins. The arrow indicates the overexpressed protein.

F1 and *P. putida* mt-2 with a BTX mixture in order to investigate whether the metabolic flux of *p*-xylene into the *tod* pathway could be prevented by the rapid uptake of *p*-xylene by the *tol* pathway. However, a significant amount of 3,6-dimethylcatechol (45 mg/liter) was still found to accumulate in the culture broth, which indicates that the complete mineralization of a BTX mixture cannot be obtained by coculture of the two strains.

However, when *P. putida* mt-2 was incubated with 0.5 g of BCG per liter, nearly 88% of the initially added BCG was degraded within 12 h, as shown in Fig. 1. The absence of degradation of BCG in the uninoculated medium confirmed that the degradation of BCG was due to biological activity. Incubations of *P. putida* mt-2 on TCG or XCG also gave similar results. From the results described above, it seems possible to redirect the metabolic flux of BTX from the *tod* pathway to the *tol* pathway at the level of dihydrodiol compounds.

A key enzyme in the *tol* pathway recognizing dihydrodiols as substrates. From the above results, it is reasonable to assume that there exists at least one enzyme in the *tol* pathway which can recognize dihydrodiols as substrates. This enzyme may play a critical role in the combination of the *tod* and the *tol* pathways. In order to identify this key enzyme, genetic and enzymatic analyses of each metabolic step in the *tol* pathway encoded on the TOL plasmid pWW0 were conducted.

When *E. coli* C600 possessing the plasmid pPL392 was cultivated separately on BCG, TCG, and XCG as the sole carbon source, microbial growth was found with consumption of each of the dihydrodiols. The plasmid pPL392 contains the genes encoding the *meta* pathway of the TOL plasmid pWW0. This observation indicates that only the *meta* pathway is involved in the degradation of the dihydrodiol compounds. Thus, three enzymes in the upper pathway (xylene oxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase) are not involved in the degradation of the BCG, TCG, and XCG. When a crude extract of *E. coli* C600(pPL392) was used, NAD^+ was required for the degradation of dihydrodiol compounds and NADH was formed. When the reaction was performed under anaerobic conditions, each of the dihydrodiol compounds was still attacked by the crude extract. This result implies that the oxygenases in the *meta* pathway are not involved in recognition of the dihydrodiols, since the oxygenase cannot function under anaerobic conditions. When BCG, TCG, or XCG was incubated with the crude extract of *E. coli* C600(pPL392) under anaerobic conditions, the reaction products were detected in the HPLC analysis. All reaction products were eluted at the identical retention time of 2.6 min. However, no reaction product was detected under aerobic conditions. The reaction products observed under anaerobic conditions disappeared when oxygen was supplied to the reaction mixture. This observation indicates that the reaction product may be a substrate for oxygenase. In the *meta* pathway, two oxygenases are involved: toluate 1,2-dioxygenase and catechol 2,3-dioxygenase. When the crude extract was pretreated with H_2O_2, which is known to suppress the activity of catechol 2,3-dioxygenase (16, 17), the reaction product did not disappear when oxygen was supplied. This result strongly suggests that the reaction product is a substrate of catechol 2,3-dioxygenase. On the basis of the observation that Gibbs reagent changes the color of the solution containing the reaction products to reddish brown (6), it can be assumed that the reaction products may be dihydroxy aromatic compounds. Furthermore, the retention time of the reaction products in the HPLC analysis (2.6 min) was almost identical to that of authentic catechol (2.58 min). We also analyzed authentic 3-methylcatechol and 3,6-dimethylcatechol by HPLC, and their retention times were 4.5 and 5.53 min, respectively. From the above observations, it is evident that the key enzyme recognizing the dihydrodiols in the *tol* pathway requires NAD^+ for activity and produces dihydroxy aromatic compound as a reaction product. Thus, TACG dehydrogenase in the *tol* pathway was thought to be the most likely candidate for the key enzyme in connecting the *tod* and the *tol* pathways.

To confirm this hypothesis, a crude extract of *E. coli* JM109(pTOL047) was used to examine whether TACG dehydrogenase could recognize dihydrodiols as substrates. The plasmid pTOL047 contains the *xylL* gene encoding the TACG dehydrogenase. Overexpression of the TACG dehydrogenase under control of the *tac* promoter was analyzed by SDS-PAGE. Figure 2 shows the protein profiles of the extracts obtained from JM109 cells containing either pKK223-3 or pTOL047. It can clearly be seen from Fig. 2 that pTOL047 overproduces a protein having a molecular mass of about 28 kDa, consistent with the molecular mass calculated from the amino acid sequence of TACG dehydrogenase reported by Neidle et al. (18). Incubation of IPTG-induced cells of JM109(pTOL047) with TACG resulted in the formation of 4-methylcatechol, which confirmed that the overexpressed pro-

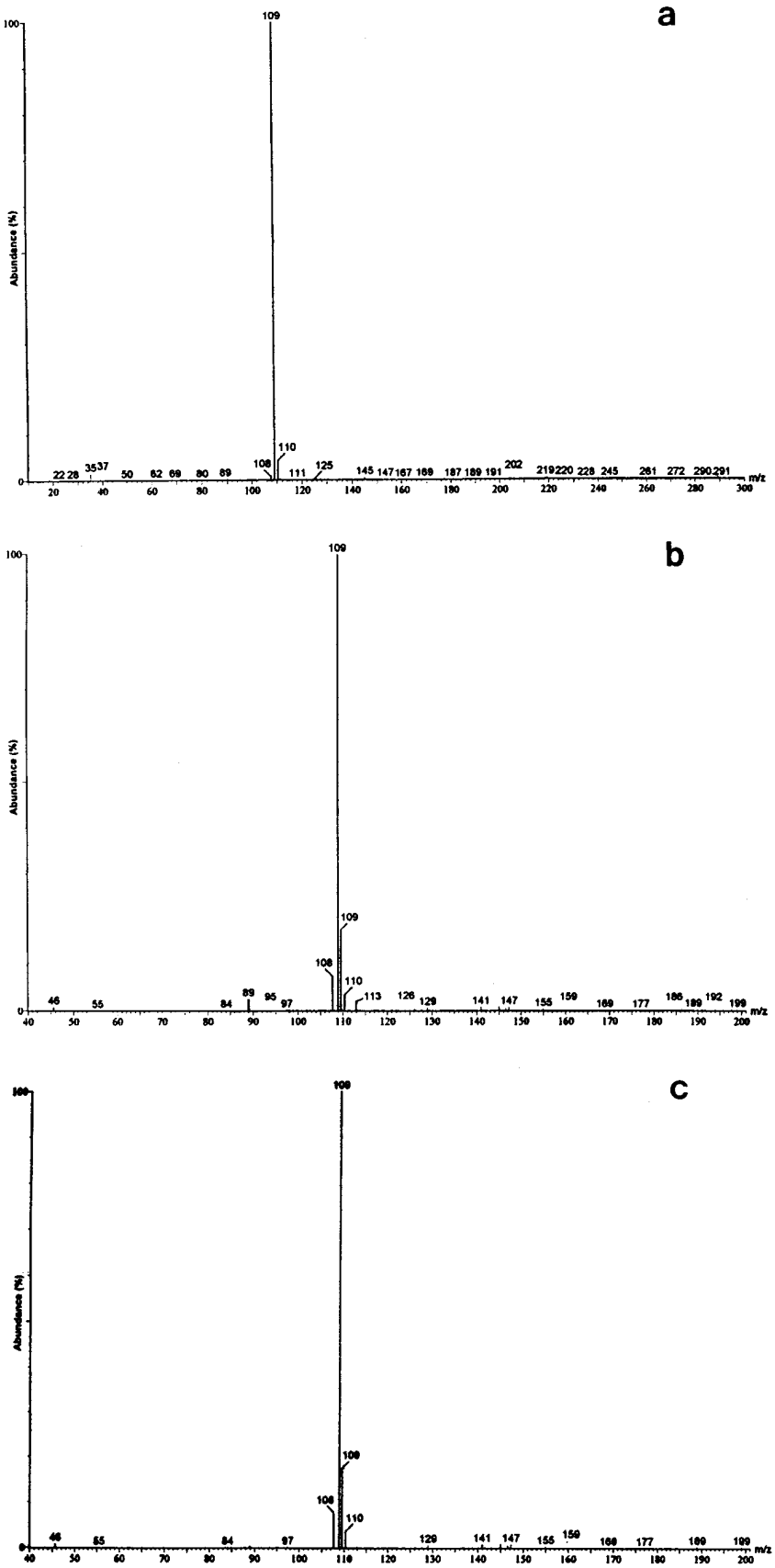

FIG. 3. Mass spectra of the reaction products of BCG (a), TCG (b), and XCG (c) obtained with crude extract of *E. coli* JM109 containing pTOL047, which overproduces TACG dehydrogenase.

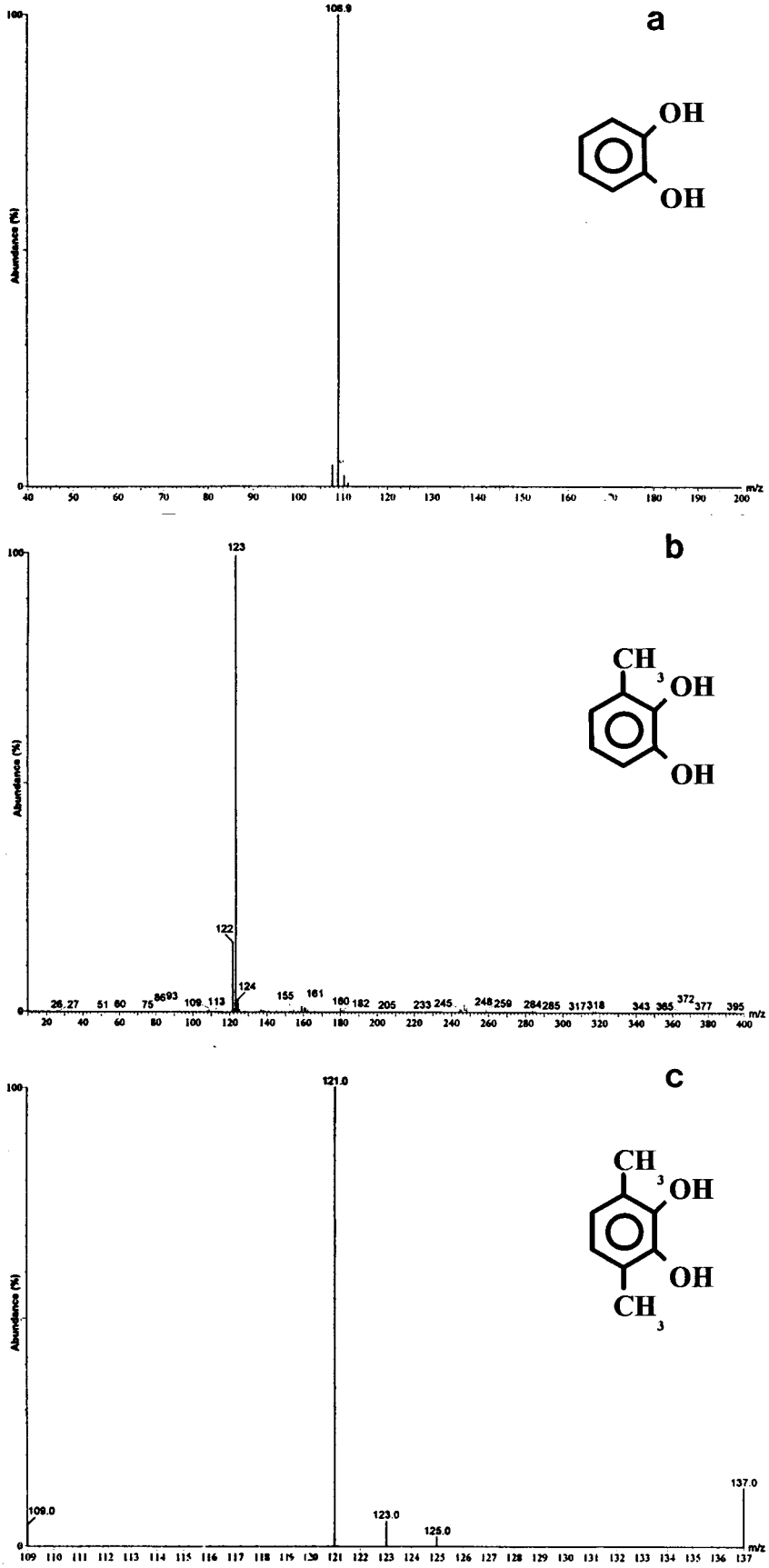

FIG. 4. Mass spectra of authentic samples of catechol (a), 3-methylcatechol (b), and 3,6-dimethylcatechol (c).

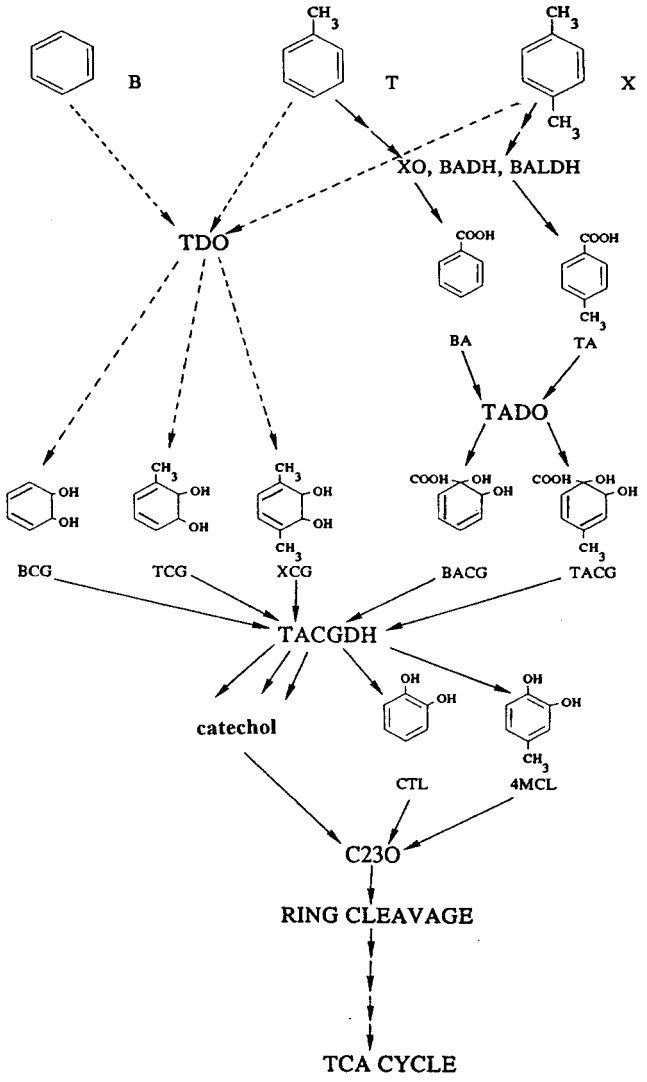

FIG. 5. Metabolic pathways constructed for the simultaneous degradation of components of a BTX mixture. Dotted lines represent the *tod* pathway, and solid lines represent the *tol* pathway. Abbreviations: B, benzene; T, toluene; X, *p*-xylene; TDO, toluene dioxygenase, XO, xylene oxidase; BADH, benzyl alcohol dehydrogenase; BALDH, benzaldehyde dehydrogenase; BA, benzoic acid; TA, *p*-toluic acid; TADO, toluate dioxygenase; BACG, *cis*-benzoate-dihydrodiol; TACGDH, *cis*-*p*-toluate-dihydrodiol dehydrogenase; CTL, catechol; 4MCL, 4-methylcatechol; C23O, catechol 2,3-dioxygenase; TCA, tricarboxylic acid.

tein is TACG dehydrogenase. The crude extract of IPTG-induced *E. coli* JM109(pTOL047) was incubated with dihydrodiols in the presence of NAD^+. As expected, all three dihydrodiol compounds (BCG, TCG, and XCG) were attacked, resulting in the formation of the reaction products. Crude extract of *E. coli* JM109(pKK223-3) used as a control showed no conversion of dihydrodiols. HPLC analyses of the reaction mixtures showed that all of the reaction products were eluted at the identical retention time of 2.6 min. In order to identify each of the reaction products in more detail, the reaction mixture was extracted with methylene chloride and after evaporation, the residual powder was dissolved in 1 ml of methanol and analyzed by LC/MS. The molecular ion peaks of three reaction products were detected at an *m*/*z* value of 109, as shown in Fig. 3. Since the LC/MS system used in this work generates a negatively charged molecular ion with a mass 1

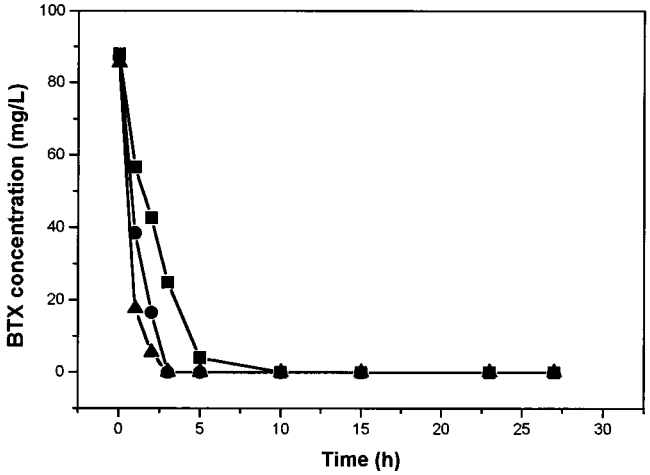

FIG. 6. Typical profiles of biodegradation of BTX mixture by *P. putida* TB105. Symbols: ■, benzene; ●, toluene; ▲, *p*-xylene.

unit less than the molecular weight, the *m*/*z* value of 109 corresponds to the molecular ion peak of catechol, which has a molecular weight of 110. We also analyzed authentic samples of catechol, 3-methylcatechol, and 3,6-dimethylcatechol by LC/MS under the same conditions. As shown in Fig. 4, molecular ion peaks of these standard compounds appeared at *m*/*z* values which are 1 unit less than their molecular weights. Detailed studies of the action of TACG dehydrogenase are in progress in our laboratory. It can, however, be proposed that TACG dehydrogenase is a key enzyme which recognizes dihydrodiols originating from the *tod* pathway as substrates.

Construction of a hybrid strain that mineralizes BTX mixtures. Vertical expansion of catabolic pathways requires the addition of a series of metabolic steps to a preexisting pathway in order to generate a hybrid one (20, 22). *P. putida* mt-2 is able to grow on toluene and *p*-xylene as a carbon source because of the *tol* pathway but cannot grow on benzene because it lacks enzyme machinery for catabolism of benzene. *P. putida* mt-2, however, was observed to attack dihydrodiols formed from benzene, toluene, and *p*-xylene by toluene dioxygenase in the *tod* pathway and to mineralize these dihydrodiols. On the basis of the above observations, the plasmid pTOD037, which possesses the genes encoding toluene dioxygenase, was introduced into *P. putida* mt-2 in order to construct a hybrid strain which can mineralize BTX mixtures. The resulting transformant was isolated on LB agar plates containing indole and streptomycin and designated *P. putida* TB105. The proposed metabolic pathway for BTX degradation in *P. putida* TB105 is represented in Fig. 5: each compound in the BTX mixture is oxidized to corresponding dihydrodiols by the action of the toluene dioxygenase, and the resulting dihydrodiols are fluxed into the *tol* pathway at the level of TACG dehydrogenase. Portions of toluene and *p*-xylene can also be metabolized through the original *tol* pathway.

The ability of *P. putida* TB105 to simultaneously mineralize components of a BTX mixture was examined, and the results obtained are shown in Fig. 6. The specific rates of degradation of BTX by *P. putida* TB105 were found to be about 40, 84, and 96 mg of compound per g of biomass per h for benzene, toluene, and *p*-xylene, respectively. In order to investigate whether any metabolic intermediates are accumulated in the culture broth, analysis of the culture broth was performed by UV/VIS spectrophotometry and HPLC. The absorbance spec-

trum of the culture broth over the wavelength from 210 to 700 nm showed that there is no accumulation of metabolic intermediates, because no peak was detected (data not shown). When the culture broth was analyzed by HPLC, no metabolic intermediate including dihydrodiol compounds produced from the *tod* pathway was detected. To examine whether the volatile intermediate is produced, the headspace was analyzed by gas chromatography, but no peak was found except for unconsumed benzene, toluene, and *p*-xylene. Therefore, we concluded that *P. putida* TB105 constructed in this work mineralizes the three compounds of a BTX mixture simultaneously without accumulating any metabolic intermediate.

The metabolic pathway engineering attempted in this work is expected to make a significant contribution to the construction of microorganisms with a great potential for the bioremediation of pollutants.

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