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Toluene degradation pathway from *Pseudomonas putida* F1: substrate specificity and gene induction by 1-substituted benzenes

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The metabolism of *n*-alkylbenzenes (C_3–C_7), biphenyl, styrene and cumene by the *tod* pathway from *Pseudomonas putida* F1 was examined in terms of catabolism by the pathway enzymes and the inducibility of the *tod* operon. F1 cells grown on toluene exhibited oxygen consumption in the presence of the compounds examined. Toluene dioxygenase (TDO) catalyzed the formation of monol, *cis*-dihydrodiol and triol metabolites from the *n*-alkylbenzenes tested and the triol formed from *n*-propylbenzene was metabolized to the derivative, 2-hydroxy-6-oxohexa-2,4-dienoate (HOHD), by subsequent enzymes in the *tod* pathway. Biotransformation of the tested compounds with toluene-grown F1 cells resulted in the accumulation of ring cleavage HOHD derivatives; the metabolites were inefficiently metabolized by cell extracts of toluene-grown F1 cells, indicating that 6-methyl-HOHD hydrolase encoded by *todF* might be a determinant for the further degradation of the selected 1-substituted benzenes. The results obtained from enzyme activity assays and reverse transcription polymerase chain reaction (RT-PCR) showed that not only growth-supporting substrates, but also *n*-propylbenzene, styrene and cumene act as inducers of the *tod* operon. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 163–170.

Keywords: *tod* pathway; monoalkylbenzenes; toluene dioxygenase; cometabolism; *Pseudomonas putida* F1

Introduction

Microorganisms play a major role in biogeochemical cycling on Earth by virtue of their diverse metabolic capabilities, ubiquity and fast growth. They degrade natural organic compounds and readily degrade many xenobiotic compounds. In many cases, the incomplete degradation of xenobiotic organic compounds by microorganisms is initiated by preexisting degradation systems which have a relaxed gene induction and a relaxed substrate specificity. This cometabolism or cooxidation appears to be of great importance in removing hazardous chemicals from contaminated sites [14,28].

Pseudomonas putida F1 utilizes benzene, toluene and ethylbenzene as carbon and energy sources for growth [8–10]. The enzymatic pathway for conversion of these aromatic hydrocarbons to TCA cycle intermediates is called the toluene degradation (*tod*) pathway (Figure 1) [6,11]. The pathway consists of seven enzymatic reactions. The genes responsible have been cloned, sequenced, and arranged in the order, *todFC1C2BADEGIH*, spanning ca. 9.5 kb [4,17,21,30,32]. Toluene dioxygenase (TDO; encoded by the *todC1C2BA* genes), the first enzyme of the pathway, catalyzes the formation of the respective *cis*-1,2-dihydrodiol from each growth-supporting substrate [8,9,11]. For instance, two hydroxyl groups are added at the 2,3 position of toluene to form *cis*-(1*S*,2*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) [9]. The *cis*-dihydrodiol is then dehydrogenated to 3-methylcatechol by a NAD^+-dependent *cis*-toluene dihydrodiol dehydrogenase (TodD) [24]. 3-Methylcatechol undergoes *meta*-cleavage by 3-methylcatechol-2,3-dioxygenase (TodE) to form a yellow-colored 2-hydroxy-6-oxo-6-methylhexa-2,4-dienoate (6-methyl-HOHD) which is then hydrolyzed to acetic acid and 2-hydroxypenta-2,4-dienoate by 6-methyl-HOHD hydrolase (TodF). 2-Hydroxypenta-2,4-dienoate is transformed by a series of enzymes, 2-hydroxypenta-2,4-dienoate hydratase (TodG), 4-hydroxy-2-oxovalerate aldolase (TodH) and acylating aldehyde dehydrogenase (TodI), to produce pyruvate and acetyl-CoA.

In the *tod* pathway, the first four reaction steps are critical for 1-substituted benzenes to be catabolized to the TCA cycle intermediates since the remaining three enzymatic reaction steps use the same intermediate regardless of the substitutions. Many 1-substituted benzenes such as monoalkylbenzenes [5,13,22,26], biphenyl [3,7], styrene, [29] are degraded by microorganisms which use enzyme systems similar to the *tod* pathway. However, the substrate specificities of the isofunctional enzymes in the pathways are sometimes different from each other and this results in separate degradation pathways for specific compounds. For instance, the *tod* pathway enzymes degrade biphenyl to the ring fission product, 6-phenyl-HOHD, as a dead-end metabolite. When a *bphD* gene (equivalent to *todF*) from biphenyl-growing *P. pseudoalcaligenes* KF707 was introduced to *P. putida* F1, the resulting strain was reported to grow on biphenyl [7]. This result showed that TodF functions as a critical bottleneck blocking further degradation of biphenyl by the *tod* pathway enzymes. Although extensive studies on the substrate specificity of TDO have been carried out [11,15], not much is known about the substrate specificities of the next three enzymes in the *tod* pathway.

In this study, we examined the possibility of whether the *tod* pathway enzymes could cometabolize other 1-substituted ben-

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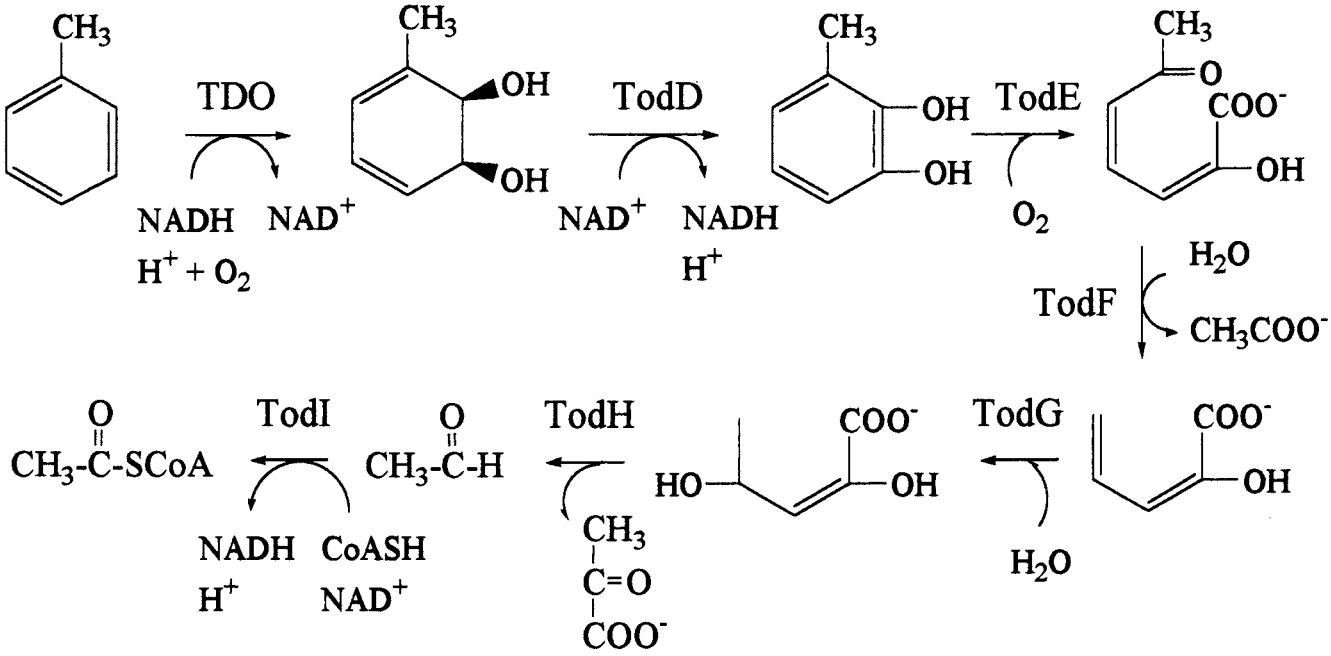

Figure 1 Toluene degradation (*tod*) pathway from *P. putida* F1. The abbreviations for enzymes are described in the text.

zenes. Our primary interest was to determine how structural changes in the side chain on the benzene ring affect their catabolism by *tod* pathway enzymes and their induction of the *tod* operon. For this purpose, we chose *n*-alkylbenzenes with differing chain lengths and other compounds such as biphenyl, cumene and styrene to change the functional group on the benzene ring. Our results show that the first three enzymes in the *tod* pathway have relaxed substrate specificities and the *tod* operon also has a relaxed effector specificity mechanism. The results obtained could provide the basis for metabolic engineering of the *tod* pathway to degrade the non-growth-supporting 1-substituted aromatic hydrocarbons to TCA cycle intermediates.

Materials and methods

Materials

Most compounds used in this study were obtained from Aldrich Chemical Co. (Milwaukee, WI) or from Sigma Chemical Co. (St. Louis, MO). Exceptions were isopropyl-β-D-thiogalactopyranoside (IPTG; Duchefa, Haarlem, the Netherlands) and 2,3-dihydroxybiphenyl (Wako Pure Chemicals, Japan). All chemicals were of analytical grade. *P. putida* F1 [11] and *Escherichia coli* JM109(pDTG601A) [31] were provided by Dr. D. Gibson of the University of Iowa.

Oxygen consumption assays

P. putida F1 was grown in minimal salts medium (MSB) [27] to a unit absorbance at the optical density at 600 nm with toluene vapour in a shaking incubator at 180 rpm and 28°C. Cells (1 ml) were harvested in 1.5 ml microcentrifuge tubes by centrifugation and stored at −72°C until used. For oxygen uptake assays, cells were resuspended in 1 ml of 50 mM Na-phosphate buffer (pH 7.0) and oxygen consumption was measured at 27°C with a Clark-type oxygen electrode (Rank Brothers, Cambridge, England) as described previously [18]. Reactions were initiated by the addition of 2 μl of a 50 mM solution of substrate in methanol. The background oxygen consumption by *P. putida* F1 in the absence of added compound was subtracted from the oxygen consumption. Oxygen consumption assays were conducted at least three times and the initial rates of oxygen consumption were determined and used for calculation of averages and standard deviations.

Biotransformation of n-alkylbenzenes

To detect metabolites accumulated through the action of *tod* pathway enzymes, *P. pudita* F1 cells grown on toluene were prepared as described in "oxygen consumption assays". Frozen cells were resuspended to an OD_{600} of 1 in 40 ml of 50 mM Na-phosphate buffer (pH 7.0) containing 20 mM glucose. Chemicals were added to the reaction mixtures to a final concentration of 0.8 mM from a 0.1 M stock solution dissolved in methanol. Biotransformations were conducted in 250 ml Erlenmeyer flasks at 28°C with shaking at 180 rpm for 4 h. To each supernatant, an internal standard of naphthalene dissolved in methanol was added to a final concentration of 0.4 mM. The mixtures were extracted with NaOH-washed ethyl acetate, which were subsequently dried over anhydrous sodium sulfate and concentrated to approximately 30 μl under nitrogen gas. To identify products formed by TDO from *n*-alkylbenzenes, *E. coli* JM109(pDTG601A), which contains the cloned *todC1C2BA* genes encoding the TDO components ($Oxygenase_{TOL}$, $Ferredoxin_{TOL}$ and $Reductase_{TOL}$) from *P. putida* F1 [31], was used for the biotransformation. *E. coli* JM109(pDTG601A) cells expressing TDO were prepared by IPTG treatment as described previously [19] and were resuspended to an OD_{600} of 1 in 100 ml of 50 mM Na-phosphate buffer (pH 7.0) containing 20 mM glucose and

0.05% (v/v) chemical. Biotransformations were conducted in 500 ml Erlenmeyer flasks at 28°C with shaking at 180 rpm for 48 h. Each supernatant was extracted with NaOH-washed ethyl acetate as described previously [23]. The organic extracts were dried over anhydrous sodium sulfate and concentrated by rotary evaporation at 35°C under reduced vacuum. Phenyl boronic acid (PBA) and methane boronic acid (MBA) derivatives of *cis*-diols were prepared in ethyl acetate for gas chromatography mass spectrometry (GC-MS) analysis as described previously [12,23].

Biotransformation of HOHD derivatives with cell extracts

Cells grown from 100 ml of MSB medium with toluene were suspended in 2 ml of lysis buffer (50 mM Na-phosphate (pH 6.0), 5% glycerol, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 40 μg of DNase and 1 mg of lysozyme) and cells were disrupted on ice by sonication (Sonoplus HD2200 ultrasonic homogenizer; Bandelin, Germany). The homogenate was centrifuged at 14,000×*g* for 30 min at 4°C. The supernatant was used as the extract. HOHD derivatives were made as described above, but the chemicals were supplied in the vapor phase except biphenyl which was added as a solid (20 mg) and glucose was omitted from the suspension buffer. After biotransformation, cells were removed by centrifugation and the supernatants were used as the source of HOHD derivatives. Yellow color in excess was diluted with 50 mM Na-phosphate buffer (pH 7.0). TodF activity toward HOHD derivatives was measured at 25°C by following disappearance of the yellow substrate on a spectrophotometer (model 2130; Scinco Co. Ltd, Korea). The assay mixtures contained 1 ml of 50 mM Na-phosphate buffer (pH 7.0), ring fission product, 40 μg of crude cell extract. Absorbances used to monitor the change of concentration of 6-propyl-, 6-butyl-, 6-isopropyl-, 6-vinyl- and 6-phenyl-HOHD were 395, 395, 395, 419 and 434 nm, respectively.

Analysis, purification and identification of metabolites

Analytical thin-layer chromatography (TLC) of extracts was performed on silica gel 60 F_{254} sheets (0.2 mm thickness; Merck, Gibbstown, NJ) with chloroform–acetone (8:2) as a developing solvent. Preparative TLC was carried out on silica gel (0.5 mm thickness; Merck) and metabolites were visualized under UV light and were purified from the silica gel plates as described previously [23]. GC-MS was performed using a Hewlett-Packard model 6890 Plus GC equipped with a Hewlett-Packard Ultra-1 capillary column (0.2 mm×25 m; film thickness 0.33 μm). The column temperature was initially kept at 70°C for 5 min and then programmed from 70°C to 280°C at 10°C/min with a helium flow of 25 cm/s. Temperatures of the injection port and transfer line were 220°C and 280°C, respectively. Samples (1 μl each) were injected at a split ratio of 50:1, and mass spectra were obtained using a Hewlett-Packard model HP5973 mass selective detector with electric impact ionization (70 eV). Proton (^{1}H) nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity Plus 300 at 300.0 MHz. Chemical shifts (δ) are reported in parts per million with respect to tetramethylsilane, and coupling constants (*J* values) are given in Hertz. ^{1}H NMR determinations were carried out by Korea Basic Science Institute (Pusan Branch, Korea).

Assays of TodE activity with a resting cell system

P. putida F1 was grown in MSB with 5 mM succinate and each compound in vapour for 48 h at 28°C. Cells were harvested by centrifugation and suspended in 10 mM Na-phosphate buffer to an OD_{600} of 0.5. An amount of 20 μl of 50 mM 2,3-dihydroxybiphenyl in methanol was added to 10 ml of the cell suspension in 250 ml Erlenmeyer flasks. The reaction mixtures were incubated at 25°C with shaking at 130 rpm for 30 min and the supernatants (1 ml each) were obtained by centrifugation. Absorbance at 434 nm was recorded from the supernatants to determine the amount of 6-phenyl-HOHD formed. The rate of product formation was linear during the incubation period. The molar extinction coefficient used for the product was 19,800 cm^{-1} M^{-1} at the wavelength given [25].

RNA preparation and reverse transcription (RT)

Total RNA from each culture obtained for assays of TodE activity with a resting cell system was purified by using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. The resulting RNA (0.5 μg) in a 20-μl reaction volume was incubated with RNase-free DNase (1 U, Promega) and RNasin (80 U, Promega) for 30 min at 37°C followed by heat inactivation of DNase at 70°C for 30 min. Reverse transcription (RT) reactions were performed with the Promega Reverse Transcription System (Promega A3500). The components of each reaction mixture were avian myeloblastosis virus (AMV) reverse transcriptase (40 U), dNTP (1 mM), $MgCl_2$ (5 mM), RNasin (20 U), a reverse primer (todFR; 0.25 μM), 0.5 μg RNA in the buffer supplied by the manufacturer. Reaction mixtures were incubated for three cycles of 15 min at 25°C followed by 15 min at 42°C. At the end of the reaction, the reaction mixtures were boiled for 5 min and kept on ice for 10 min.

RT polymerase chain reaction (PCR) of the todF gene

The reaction mixtures (20 μl) contained 4 μl of c-DNA formed in the RT reaction, todFR primer (0.5 μM), todFF primer (0.5 μM) and Ex-Taq DNA polymerase (1 U) in the buffer supplied by the manufacture (TaKaRa, Japan). The positive control contained chromosomal DNA (20 ng) in place of c-DNA in the same reaction conditions. PCR reactions were carried out with a Biometra personal cycler (Biotron, Germany). The standard PCR conditions were 30 cycles of 94°C for 30 min, 55°C for 30 s and 72°C for 1 min. The primers of todFF and todFR were designed for amplification of the *todF* gene and its flanking regions (total 903 bp) and their sequences were 5′-TTTAAATCCCAAGGG-CACGGAGCGTAAG-3′ and 5′-TGACGTGCTTTCAGAACG-GATGGTCATT-3′, respectively. The primers were synthesized by the Bioneer Co. (Korea). Electrophoresis was conducted on a 1.2% agarose gel with TAE buffer [20]. The PCR product was purified from an agarose gel following electrophoresis with the GFX PCR DNA and Gel Band Purification Kit (Amersham-Pharmacia Biotech, Sweden). The purified DNA fragment was ligated into the pGEM-T easy vector from Promega and the resulting plasmid was amplified in *E. coli* JM109. The inserted DNA was sequenced with M13 forward and reverse primers by using the Applied Biosystems Inc. model Prism 377. Sequence determinations were carried out by the Korea Basic Science Institute. In addition, to detect any DNA contamination in the RNA preparations, PCR was carried out with the RNA samples as

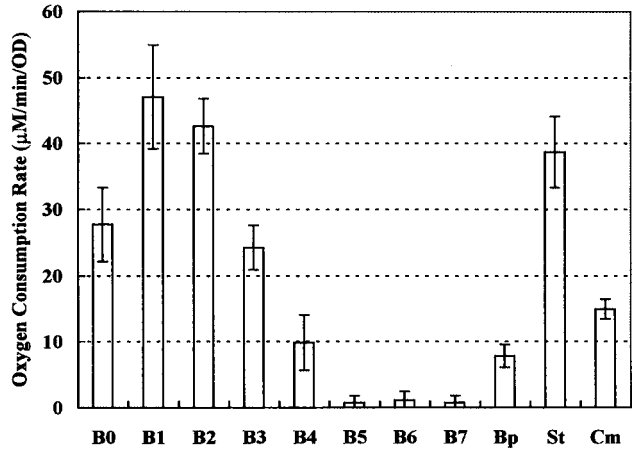

Figure 2 Initial rates of oxygen consumption in the presence of various chemicals by *P. putida* F1 grown on toluene. Values have been normalized to the cell density (OD_{600}). Details of experimental procedures are given in *Materials and methods*. Abbreviations for chemicals: B0, benzene; B1, toluene; B2, ethylbenzene; B3, *n*-propylbenzene; B4, *n*-butylbenzene; B5, *n*-pentylbenzene; B6, *n*-hexylbenzene; B7, *n*-heptylbenzene; Bp, biphenyl; St, styrene; Cm, cumene.

follows. The components of reaction mixtures (20 μl) contained Ex-Taq DNA polymerase (1 U), dNTP (0.2 mM), todFR primer (0.5 μM), todFF primer (0.5 μM) and RNA (0.2 μg) in the buffer supplied by the manufacturer. The standard PCR conditions were the same as described above.

Results

Oxygen consumption by P. putida F1 cells grown on toluene

In order to determine whether *n*-alkylbenzenes (C_3–C_7), biphenyl, styrene and cumene could be degraded by *tod* pathway enzymes, we examined oxygen consumption by toluene-grown cells in the presence of the compounds. The resulting initial rate of oxygen consumption could be determined primarily by TDO since the first reaction step is crucial for the overall metabolism of the substrates by the rest of the *tod* pathway enzymes. The result of initial rates of oxygen consumption by cells grown on toluene is shown in Figure 2. In addition to growth-supporting substrates (e.g. benzene, toluene and ethylbenzene), incubation of the cells with *n*-propylbenzene, *n*-butylbenzene, biphenyl, styrene or cumene resulted in high oxygen consumption. Furthermore, incubation with *n*-pentyl-, *n*-hexyl-, or *n*-heptylbenzene resulted in low levels of oxygen consumption. However, incubation with *n*-octyl-, *n*-nonyl- or *n*-decylbenzene did not result in oxygen consumption.

Accumulation of dead-end metabolites from non-growth substrates by tod pathway enzymes

To determine the metabolic barrier that prevents complete mineralization of the non-growth-supporting 1-substituted benzenes, the accumulation of dead-end products from the compounds was examined with F1 cells grown on toluene. Cell suspensions incubated with *n*-propylbenzene and *n*-butylbenzene were very yellow, indicating the presence of a *meta*-cleavage product [10]. However, cell suspensions incubated with *n*-alkylbenzenes (C_5–C_7) did not yield visible color during the incubation. In addition, cell suspensions incubated with biphenyl, styrene and cumene showed a deep yellow color. GC-MS analysis was conducted with organic extracts and MBA-treated organic extracts to identify the metabolites that accumulated besides the ring fission product. The organic extracts from the reaction mixtures of *n*-alkylbenzenes (C_3–C_7), biphenyl, styrene and cumene did not yield GC peaks having molecular ions expected for catechols [$(M_{hydrocarbon}+32)^+$] or MBA derivatives of *cis*-dihydrodiols [$(M_{hydrocarbon}+58)^+$]. However, concentrated organic extracts from *n*-alkylbenzenes (C_5–C_7) were yellow and yielded GC peaks at low levels (5–20% of added naphthalene) with a molecular ion of $(M_{hydrocarbon}+16)^+$, which was shown to be benzylic monooxygenated products with the fragment peaks at *m*/*z* 79 and 107 from the mass analysis. The identities of these products were confirmed by ^{1}H NMR analysis as described in Tables 1 and 2. No products were detected from the same incubations with cells grown on succinate during the incubation period. Cell extracts of toluene-grown cells had no activity toward ring fission derivatives formed from biphenyl, *n*-propylbenzene and *n*-butylbenzene, and had a trace level activity toward ring fission derivatives formed from cumene and styrene

Table 1 Analytical TLC and GC-MS properties of major compounds formed from *n*-alkylbenzene by *E. coli* JM109(pDTG601A) expressing TDO

			GC/MS data[b]	
Substrate[a]	Compound	TLC R_f	R_t (min)	Ion fragment *m*/*z* values (percent relative intensity)
B3	I	0.31	24.01 (PBA)	240 (M^+ 50), 211 (28.3), 197 (100), 107 (36.2), 77 (23.9)
B4	I	0.33	24.8 (PBA)	254 (M^+ 42), 211 (24.6), 197 (100), 107 (39.1), 77 (24.6)
	II	0.65	16.0	150 (M^+ 12.9), 107 (100), 79 (47.8), 44 (64)
B5	I	0.36	25.9 (PBA)	268 (M^+ 37.7), 211 (23.2), 197 (100), 107 (55.8), 77 (25.4)
	II	0.67	17.47	164 (M^+ 8.7), 107 (100), 79 (38.4), 44 (18.9)
B6	I	0.37	26.87 (PBA)	282 (M^+ 40.6), 211 (24.6), 197 (100), 107 (50.7), 77 (23.2)
	II	0.69	18.39	178 (M^+ 7.3), 107 (100), 79 (31.9)
B7	I	0.39	27.25 (PBA)	296 (M^+ 39.1), 211 (26.1), 197 (100), 107 (23.9), 77 (20.2)
	II	0.70	19.10	192 (M^+ 5.0), 107 (100), 79 (28.3)
PP	I	0.07	24.90 (PBA)	256 (M^+ 14.5), 209 (89.5), 149 (97.7), 105 (84.4), 77 (100), 57 (86)

[a]Abbreviations for chemicals are the same as in Figure 2 with PP for 1-phenyl-1-propanol.
[b]R_t values and ion fragment patterns were determined under conditions described in the text. (PBA) indicates that the diols were analyzed by GC-MS as PBA derivatives.

Table 2 ^{1}H NMR properties of major compounds formed from *n*-alkylbenzene by *E. coli* JM109(pDTG601A) expressing TDO

Substrate[a]	Compound	^{1}H NMR chemical shifts (δ)[b]	Identification or possible structure
B3	I	5.91–5.96 (m, 1H), 5.66–5.71 (m, 2H), 4.22 (m, 1H), 3.88 (d, *J*=5.9, 1H), 2.27–2.10 (m, 2H), 1.46–1.61 (m, 2H), 0.94 (t, *J*=7.3, 3H)	*cis*-3-propyl-3,5-cyclohexadiene-1,2-diol
B4	I	5.85–5.91 (m, 1H), 5.66–5.71 (m, 2H), 4.20–4.24 (m, 1H), 3.89 (d, *J*=5.9, 1H), 2.12–2.31 (m, 2H), 1.28–1.57 (m, 4H), 0.93 (t, *J*=7.1, 3H)	*cis*-3-butyl-3,5-cyclohexadiene-1,2-diol
	II	7.21–7.31 (m, 5H), 4.59 (t, *J*=6.8, 1H), 1.24–1.76 (m, 4H), 0.92 (t, *J*=7.4, 3H)	1-phenyl-1-butanol
B5	I	5.85–5.90 (m, 1H), 5.65–5.80 (m, 2H), 4.19–4.23 (m, 1H), 3.89 (d, *J*=5.9, 1H), 2.10–2.30 (m, 2H), 1.25–1.58 (m, 6H), 0.89 (t, *J*=7.0, 3H)	*cis*-3-pentyl-3,5-cyclohexadiene-1,2-diol
	II	7.18–7.31 (m, 5H), 4.56 (t, *J*=6.7, 1H), 1.60–1.88 (m, 2H), 1.19–1.41 (m, 4H), 0.88 (t, *J*=7.1, 3H)	1-phenyl-1-pentanol
B6	I	5.86–5.92 (m, 1H), 5.67–5.71 (m, 2H), 4.21–4.24 (m, 1H), 3.90 (d, *J*=5.9, 1H), 2.14–2.27 (m, 2H), 1.29–1.57 (m, 8H), 0.88–0.93 (m, 3H)	*cis*-3-hexyl-3,5-cyclohexadiene-1,2-diol
	II	7.20–7.33 (m, 5H), 4.58 (t, *J*=6.7, 1H), 1.65–1.89 (m, 2H), 1.23–1.44 (m, 6H), 0.86–0.91 (m, 3H)	1-phenyl-1-hexanol
B7	I	5.86–5.92 (m, 1H), 5.67–5.71 (m, 2H), 4.22–4.24 (m, 1H), 3.90 (d, *J*=5.9, 1H), 2.14–2.23 (m, 2H), 1.12–1.61 (m, 10H), 0.88–0.93 (m, 3H)	*cis*-3-heptyl-3,5-cyclohexadiene-1,2-diol
	II	7.23–7.32 (m, 5H), 4.58 (t, *J*=6.7, 1H), 1.65–1.75 (m, 2H), 1.20–1.38 (m, 8H), 0.86–0.90 (m, 3H)	1-phenyl-1-heptanol
PP	I	5.90–5.97 (m, 2H), 5.71–5.77 (m, 1H), 4.26–4.30 (m, 1H), 4.07–4.14 (dd, *J*=7.0, 12.8, 1H), 4.00–4.05 (m, 1H), 1.53–1.89 (m, 2H), 0.14 (m, 3H)	*cis*-3-(1′-hydroxypropyl)-3,5-cyclohexadiene-1,2-diol

[a]Abbreviations for chemicals are the same as in Figure 2 with PP for 1-phenyl-1-propanol.
[b]Determined at 300 MHz in deuterated methanol. Chemical shift multiplicities are abbreviated as follows: s, singlet; d, doublet; dd, doublet of doublet; t, triplet; m, multiplet. Coupling constants (*J* values) are given in Hertz.

with absorbance decreases of 0.036 and 0.056, respectively, for a 10-min reaction. These results showed that the *tod* pathway enzymes recognized *n*-alkylbenzenes (C_3–C_7), biphenyl, styrene and cumene, and the substrates were metabolized to the yellow *meta*-cleavage products, derivatives of HOHD.

Biotransformation of n-alkylbenzenes by E. coli JM109(pDTG601A) expressing TDO

Although *cis*-dihydrodiol products from the growth-supporting *n*-alkylbenzenes (toluene and ethylbenzene) have been reported [8,9], detailed information on the reaction of TDO with *n*-alkylbenzenes with an alkyl chain longer than C_3 is not available. IPTG-induced cells of *E. coli* JM109(pDTG601A) were incubated with *n*-alkylbenzenes as described in *Materials and methods*. During the incubation, *n*-alkylbenzenes with a chain length of C_3–C_5 were all utilized. However, the amounts of substrate conversion for *n*-hexylbenzene and *n*-heptylbenzene were 71% and 36%, respectively, based on the integration of total ion current peak areas. An example of the identification of the metabolites formed during the transformation is described for *n*-propylbenzene as follows. The cell suspension with *n*-propylbenzene yielded 22 mg of a crude ethyl acetate-extractable brown oil. Analytical TLC showed a major polar product with an R_f value of 0.31 (compound I) and two minor products with R_f values of 0.61 and 0.07. The products were purified by preparative TLC and gave 10, 1 and 0.1 mg, respectively. GC-MS analysis showed that the compound with an R_f of 0.61 was identical to 1-phenyl-1-propanol by comparing their GC-MS properties such as retention times and fragmentation patterns with authentic 1-phenyl-1-propanol. GC-MS analysis showed that compound I gave two broad peaks with retention times of 16.0 and 16.5 min showing molecular ions at *m*/*z* 136. The PBA derivative, however, gave a single peak with a molecular ion at *m*/*z* 240, and its presence coincided with the loss of the two peaks formed from compound I in the GC elution profile. This indicated that the compound was dehydrated in the GC inlet and the molecular mass of compound I was 154. From ^{1}H NMR analysis, compound I was identified as *cis*-3-propyl-3,5-cyclohexadiene-1,2-diol (Table 2). The compound, with an R_f of 0.07, gave broad peaks at 17.0–18.5 min with a molecular ion at *m*/*z* 152 by GC-MS analysis, indicating that the compound was also dehydrated in the GC inlet. This compound was formed from 1-phenyl-1-propanol, not from *cis*-3-propyl-3,5-cyclohexadiene-1,2-diol. The product formed from 1-phenyl-1-propanol was purified by preparative TLC, and GC/MS and ^{1}H NMR analysis identified the compound as a triol, *cis*-3-(1′-hydroxypropyl)-3,5-cyclohexadiene-1,2-diol (Tables 1 and 2). The triol was further

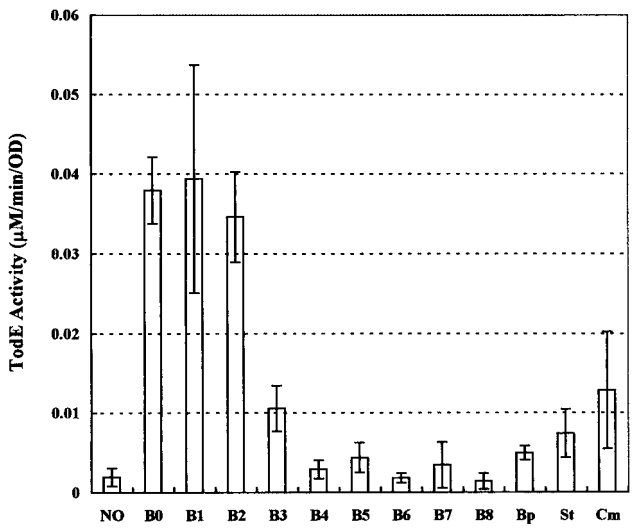

Figure 3 Induction of the *todE* gene by various chemicals. Induction levels were determined by the expression of TodE as a reporter enzyme. Values have been normalized to the cell density (OD_{600}). Details of experimental procedures are given in *Materials and methods*. NO indicates no chemical was added. Other abbreviations for chemicals are the same as in Figure 2, with B8 for *n*-octylbenzene.

metabolized to the yellow compound by cells grown on toluene, but not by cells grown on succinate.

From the analogous analyses, TDO from *P. putida* F1 catalyzed the oxidation of *n*-alkylbenzenes (C_3-C_7) to *cis*-2,3-dihydrodiol (compound I, major) and benzylic monooxygenated (compound II, minor) products (Tables 1 and 2) with relative yields of 5–8 to 1 by integration of the total ion current peak areas. In addition, TLC analysis showed that the *n*-alkylbenzenes tested were oxidized to compounds with R_f values of 0.07–0.09, indicating that triols were also formed from those compounds.

Inducibility of the tod operon

It is important to know how variations in aromatic hydrocarbon structure influence inducibility of the *tod* operon. *n*-Alkylbenzenes and other 1-substituted benzenes were tested for their ability to induce the *tod* operon. It was shown previously that 2,3-dihydroxybiphenyl was a substrate for TodE and the yellow-colored *meta* ring fission product, 6-phenyl-HOHD, was not metabolized further by the subsequent enzyme, TodF [7]. This result suggested that to determine the expression level of the *tod* operon, TodE could be used as a reporter enzyme, whose activity could be determined easily by monitoring the accumulation of a yellow ring fission product. Resting cells grown on succinate supplemented with chemical inducers were subjected to the spectrophotometric assays as described in *Materials and methods*. As expected, the growth-supporting substrates (e.g. benzene, toluene and ethylbenzene) proved to be strong inducers (Figure 3). Non-growth-supporting substrates such as *n*-propylbenzene, styrene and cumene also acted as inducers for the *tod* operon, although not as efficiently as the growth-supporting substrates.

To confirm the results obtained from the reporter enzyme assays, RT-PCR was carried out. The results obtained from the biotransformations described above indicate that TodF appears to be critical for catabolism of 1-substituted benzenes. For this reason, RT-PCR was carried out to detect transcription of the *tod* operon by amplification of the *todF* gene. Although the PCR conditions were not optimized to differentiate the expression levels, the RT-PCR result confirmed the role of each chemical in the induction of the *tod* operon as determined by the TodE activity assays. PCR products of *ca.* 900 bp were observed from cells grown on succinate supplemented with benzene, toluene, ethylbenzene, *n*-propylbenzene, styrene and cumene (Figure 4). In addition, on occasion, a very weak DNA product band was also observed from cells grown on succinate supplemented with biphenyl. The RT-PCR DNA product formed from the cells supplemented with toluene was cloned in the pGEM-T easy vector and the product identity was confirmed by DNA sequencing. Another set of experiments yielded the same RT-PCR result obtained above. Furthermore, no PCR product was observed by PCR amplification of purified RNA samples with the same primers, indicating that DNA was removed from the RNA preparations.

A ring fission product accumulated in the medium from cells grown on MSB supplemented with succinate plus *n*-butylbenzene, *n*-pentylbenzene or biphenyl after an incubation period of 3 days. This result suggests that the *tod* operon was expressed at basal levels under these culture conditions.

Discussion

TDO catalyzes the formation of enantiomerically pure *cis*-1,2-dihydroxydihydrodiols from a variety of benzene derivatives [2,11,15]. The metabolites are an important class of compounds used as chiral synthons for the synthesis of value-added compounds and biologically active compounds such as conduritols, inositols, pinitols, deoxysugar analogs and alkaloids [15]. Thus, TDO is an industrially important enzyme and studies on the substrate specificity of TDO are worthwhile.

It was shown in this study that *n*-alkylbenzenes with an alkyl chain of C_3-C_7 were substrates for TDO. The initial rate of oxygen consumption by *P. putida* F1 expressing the *tod* pathway enzymes was greatest in the presence of toluene and gradually decreased with increasing chain length to *n*-pentylbenzene (Figure 2). This could be due to reduced solubility, reduced uptake and/or an intrinsic property of TDO. Further studies on this aspect are required to address this question. It was reported earlier that *P. putida* 39/D, a TodD-mutant of strain F1, oxidized ethylbenzene to *cis*-3-ethyl-3,5-cyclohexadiene-1,2-diol, (+)-1-phenylethanol, and *cis*-3-(1′-hydroxyethyl)-3,5-cyclohexadiene-1,2-diol [8]. Results shown here expand the substrate specificity of TDO to include *n*-alkylbenzenes with an alkyl side chain of C_3-C_7. TDO catalyzes the

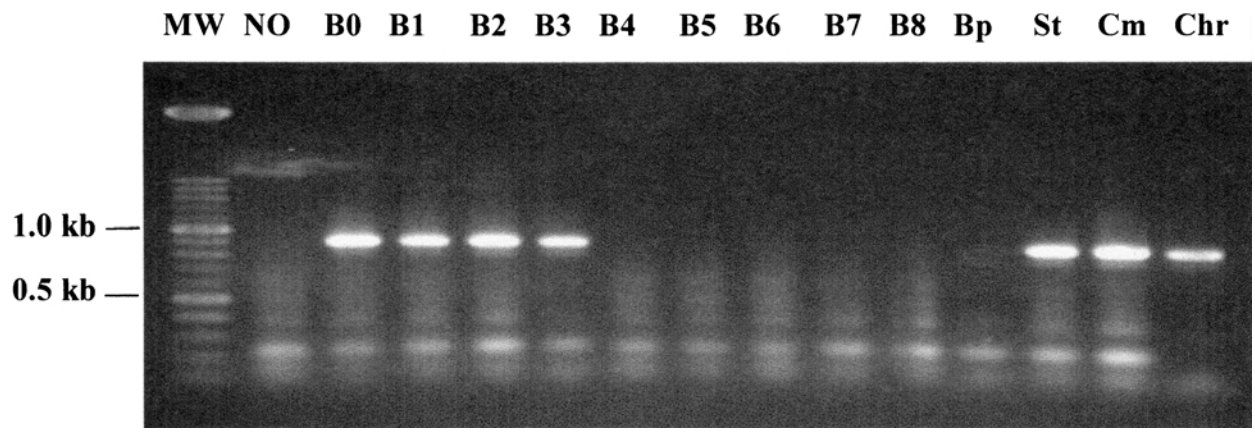

Figure 4 RT-PCR amplification of *todF* mRNA transcripts purified from *P. putida* F1 grown in 5 mM succinate supplemented with each chemical on the abscissa. Details of experimental procedures are described in *Materials and methods*. Chr represents a positive control in which the *todF* gene was amplified by PCR from chromosomal DNA. Other abbreviations for chemicals are the same as in Figure 2, with B8 for *n*-octylbenzene, and MW for 100-bp ladder.

formation of monol, *cis*-diol and triol derivatives (Tables 1 and 2). *cis*-Dihydrodiols from benzene and *n*-alkylbenzenes (C_1–C_5) formed by *P. putida* UV4 expressing TDO have been reported in a review article and the *cis*-dihydrodiol metabolites of higher homologs were not accumulated by the UV4 strain [2]. This indicates that TDO from both strains F1 and UV4 has greater activity toward benzene and *n*-alkylbenzenes with a side chain of C_1–C_4 than toward *n*-alkylbenzenes with a longer side chain.

The metabolism of 1-substituted benzenes by the *tod* pathway of *P. putida* F1 was also examined in this study. The first three enzymes in the *tod* pathway have relaxed substrate specificities that accommodate degradation of *n*-alkylbenzenes (C_3–C_7), styrene, cumene and biphenyl as well as the growth-supporting substrates. But further degradation of the non-growth chemicals was blocked by TodF. Some of these results are consistent with kinetic data obtained with purified TodF in which TodF had no detectable activity with 6-phenyl-HOHD and negligible activity with 6-isopropyl-HOHD, exhibiting 105-fold less activity than with the growth substrate, 6-methyl-HOHD [25]. Gibson *et al.* [10] reported oxygen uptake and accumulation of yellow HOHD derivatives for benzene, *n*-propylbenzene, *n*-butylbenzene and cumene from toluene-grown *P. putida* F1 cells. The study also carried out biotransformations with *n*-alkylbenzenes (C_5–C_8 and C_{10}), but the products were not detected. In addition, earlier studies [7,10] did not examine accumulation of other intermediates except HOHD derivatives. In the present study, by examining the metabolic intermediates formed by the first three enzymes in the *tod* pathway, it can be concluded that the benzylic monol such as that formed from *n*-propylbenzene by TDO is degraded to an HOHD derivative by the *tod* pathway enzymes, and TodD and TodE do not limit degradation of the compounds tested in this study.

For metabolic engineering of the *tod* pathway to degrade solvents such as *n*-propylbenzene, styrene and cumene, the induction of *tod* genes by the compounds should be determined first. Here, we show that *n*-propylbenzene, styrene, and cumene acted as inducers for the *tod* operon (Figures 3 and 4). This indicates that for the inducing compounds to be growth substrates, TodF should be able to recognize their HOHD derivatives. Interestingly, only the vinyl double bond in styrene is structurally different from the ethyl group in ethylbenzene; however, this difference caused a dramatic change in substrate recognition by TodF. We are currently carrying out random mutagenesis of the *todF* gene to identify amino acids responsible for recognition of the derivatives. In another report, it was shown that the *tod* operon was induced by various other aromatic hydrocarbons including *m*-, *p*-xylene and water-soluble JP-4 jet fuel components [1]. Although TodS and TodT encoded by the *todST* genes located at the end of the *tod* operon were proposed to be involved in transcriptional activation of the *tod* operon as a two-component signal transduction system [16], the protein component required for inducer binding has not been identified.

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