S3-3

Metabolism of 1-Substituted Benzenes by *Pseudomonas putida* F1 and Its Mutants

Kyoung Lee

Department of Microbiology, Changwon National University, Changwon-si, Kyongnam 641-773, Korea. E-mail: kLee@sarim.changwon.ac.kr

We have examined the metabolism of *n*-alkylbenzenes (C3-C7), biphenyl, styrene and cumene by the *tod* pathway from *Pseudomonas putida* F1 in terms of their catabolism by the pathway enzymes and their inducibility of the *tod* operon. F1 cells grown on toluene exhibited oxygen consumption in the presence of the chemicals examined. Toluene dioxygenase 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 of 2-hydro-6-oxohexa-2,4-dienoate (HOHD) by the subsequent enzymes in the *tod* pathway. All the chemicals tested were catabolized by F1 cells grown on toluene to ring-cleavage HOHD derivatives, indicating that 6-methyl-HOHD hydrolase encoded by *todF* was a determinant for the further degradation of the selected 1-substituted benzenes. The results obtained from the enzyme activity assay and RT-PCR showed that not only the growth-supporting substrates but also *n*-propylbenzene, styrene and cumene act as inducers of the *tod* operon. We have also isolated F1 mutants that can grow on benzene, toluene, ethylbenzene, *n*-propylbenzene, cumene and biphenyl. The genetic backgrounds of the mutants will be discussed.

Introduction. *Pseudomonas putida* F1 utilizes benzene, toluene and ethylbenzene as carbon and energy sources for growth [7,8]. The enzymatic pathway for conversion of these aromatic hydrocarbons to the TCA cycle intermediates is called the *to*luene *d*egradation (*tod*) pathway [5,9,18]. The pathway consists of a total of seven enzymatic reactions [13]. Toluene dioxygenase (TDO), the first enzyme of the pathway, has been shown to catalyze the formation of respective *cis*-1,2-dihydroxy-1,2-dihydrodiol from each growth-supporting substrate [7-9]. The *cis*-dihydrodiol then undergoes to the TCA cycle intermediates such as pyruvate and acetyl-CoA through the *tod* pathway enzymes as shown in Fig. 1.

In this *tod* pathway the first four reaction steps (upper pathway) are critical for the 1-substituted benzenes to be catabolized to the TCA cycle intermediates since the rest three enzymatic reaction steps (lower pathway) uses the same intermediate regardless of the substitutions. Many 1-substituted benzenes such as monoalkylbenzenes [4,10,14,16], biphenyl [3,6], styrene [17] are degraded by microorganisms using the enzyme systems similar to the *tod* pathway. However, the substrate specificity of the isofunctional enzymes in the pathways is sometimes different from each other and this confers discrete degradation pathways for specific chemicals. For instance, the *tod* pathway enzymes degrade biphenyl to the ring fission product of 6-phenyl-HOHD as a dead-end metabolite. When a *bphD* gene (equivalent to *todF*) from the biphenyl-growing *P. pseudoalcaligenes* KF707 was introduced to *P. putida* F1, the resultant strain was reported to grow on biphenyl [6]. This result showed that TodF functions as a critical bottleneck blocking further degradation of biphenyl by the *tod* pathway enzymes. Although extensive studies on substrate specificity of TDO have been carried out [9,11], much is not known about the substrate specificities of the next three enzymes in the *tod* upper pathway.

In this study, we have examined the possibility if the *tod* pathway enzymes could cometabolize other 1-substituted benzenes. Our primary interest was to determine how the structural changes in the side chain on the benzene ring affect their catabolism by the *tod* pathway enzymes and their inducibility of the *tod* operon. For this purpose we have chosen *n*-alkylbenzenes to change the chain length on the benzene ring and other chemicals 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 upper *tod* pathway have relaxed substrate specificities and the *tod* operon also has a relaxed effector specificity mechanism. The results obtained from this study could provide the basis for metabolic engineering of the *tod* pathway to degrade the non-growth supporting 1-substituted aromatic hydrocarbons to the TCA cycle intermediates.

Oxygen consumption by *P. putida* F1 cells grown on toluene. In order to determine whether *n*-alkylbenzenes (C3-C7), biphenyl, styrene and cumene could be degraded by the *tod* pathway enzymes, we have examined oxygen consumption by *P. putida* F1 cells grown on toluene in the presence of the chemicals. The resulting initial rate of the oxygen consumption could be determined primarily by TDO and the first reaction step is crucial for overall metabolism of the substrates by the rest *tod* pathway

enzymes. In addition to growth-supporting substrates (e.g. benzene, toluene and ethylbenzene), *n*-propylbenzene, *n*-butylbenzene, biphenyl, styrene and cumene showed high oxygen consumption. Furthermore, *n*-pentyl-, *n*-hexyl-, and *n*-heptylbenzene also showed low levels of oxygen consumption. However, *n*-octyl-, *n*-nonyl- and *n*-decylbenzene did not exhibit oxygen consumption.

Accumulation of dead-end metabolites from non-growth substrates by the *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 product from the chemicals was examined with *P. putida* F1 cells grown on toluene. Cell suspensions incubated with *n*-propylbenzene, *n*-butylbenzene were very yellow. However, cell suspensions incubated with *n*-alkylbenzenes (C5-C7) did not yielded visible color during the incubation. In addition, cell suspensions incubated with biphenyl, styrene and cumene showed deep yellow color. GC-MS analysis was conducted with organic extracts to identify the metabolites accumulated besides the ring-fission product. The organic extracts from the reaction mixtures of *n*-alkylbenzenes (C3-C7), biphenyl, styrene and cumene did not yielded GC peaks with molecular ions of $M(OH)_2^+$ of methane boronic acid and MO_2^+ which were *cis*-dihydrodiol and catechol products, respectively. However, organic extracts from *n*-alkylbenzenes (C5-C7) were yellow and yielded GC peaks at low levels (5 - 20%) with a molecular ion of MO^+, which was benzylic monooxygenated products by showing the fragment peaks at m/z 79 and 107 from the mass analysis and which were confirmed by ^{1}H-NMR analysis. No products were identified from the same incubations with *P. putida* F1 cells grown on succinate during the incubation period. These results showed that the *tod* pathway enzymes recognized *n*-alkylbenzenes (C3-C7), biphenyl, styrene and cumene, and the substrates were metabolized to the *meta*-ring cleavage product, derivative of HOHD.

Biotransformation of *n*-alkylbenzenes by *E. coli* JM109(pDTG01A) expressing TDO. Although *cis*-dihydrodiol products from the growth-supporting *n*-alkylbenzenes (toluene and ethylbenzene) have been reported [7,8], detailed information on the reaction of TDO with *n*-alkylbenzenes with an alkyl chain longer than C3 is not available. *E. coli* JM109(pDTG601A) cells expressing TDO were incubated with *n*-alkylbenzenes for biotransformation. From the TLC, GC/MS and ^{1}H-NMR analyses, TDO from *P. putida* F1 was shown to catalyze the oxidation of *n*-alkylbenzenes (C3-C7) to

cis-2,3-dihydrodiol (major) and benzylic monooxygenated (minor) products with relative yields of 5-8 to 1 by integration of total ion current peak areas. In addition, TLC analysis showed that *n*-alkylbenzenes tested were oxidized to triols which might be formed by the dioxygenation reaction of monol compounds not by the monooxygenation reaction of *cis*-diols.

Inducibility of the *tod* operon by various chemicals. It has been previously shown that 2,3-dihydroxybiphenyl was a substrate for TodE and the yellow-colored *meta* ring fission product, 6-phenyl-HOHD, was not further metabolized by the subsequent enzyme, TodF [6]. This result suggested that to determine the expression level of the *tod* operon TodE could be a reporter enzyme, whose activity could be determined easily by monitoring accumulation of a yellow ring fission product. Resting cells grown on succinate supplemented with chemical inducers were subjected to the spectrophotometric assays. As expected, the growth-supporting substrates (e.g. benzene, toluene and ethylbenzene) has been proved to be strong inducers. Interestingly, non growth-supporting substrates such as *n*-propylbenzene, styrene and cumene also act as inducers for the *tod* operon even though they are not as efficient as the growth-supporting substrates.

To further verify the results obtained from the report enzyme assays, RT-PCR has been carried out. The results obtained from biotransformation described above indicates TodF appears to be critical for the catabolism of 1-substituted benzenes. For this reason, RT-PCR was carried out to detect transcription of the *tod* operon with amplification of *todF* gene. The RT-PCR result confirmed the role of each chemical in the induction of *tod* operon as determined by the TodE activity assays. The PCR products yielding ca 900 bp DNA fragments were observed in the cells grown on succinate supplemented with benzene, toluene, ethylbenzene, *n*-propylbenzene, styrene and cumene (Fig. 2). In addition, a very weak DNA product band was also observed in the 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.

Discussing conclusions. TDO has been shown to catalyze the formation of enantiomerically pure *cis*-1,2-dihydroxydihydrodiols from a variety of benzene derivatives [2,9,11]. 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 [11]. This indicates that TDO is an industrially important enzyme and the studies on the substrate specificity of TDO are worth to continuing.

It has been reported early by Gibson and colleagues that *P. putida* 39/D, a *todD*-mutant of F1, oxidized ethylbenzene to *cis*-3-ethyl-3,5-cyclohexadiene-1,2-diol and (+)-1-phenylethanol and *cis*-3-(1′-hydroxyethyl)-3,5-cyclohexadiene-1,2-diol [7]. Our results shown here expanded the early result of the substrate specificity of TDO, that is TDO could recognize *n*-alkylbenzenes with an alkyl side chain of C3-C7 and catalyzes the formation of derivatives of monol, *cis*-diol and triols. *cis*-Dihydodiols from benzene and *n*-alkylbenzenes (C1-C5) by *P. putida* UV4 expressing TDO have been reported in a review article and the *cis*-dihydrodiol metabolites of higher homologs were not reported formed by the UV4 strain [2], indicating that there might be a size limitation on substrate acceptability between two TDOs.

It has been also examined in this study the metabolism of 1-substituted benzenes by the *tod* pathway of *P. putida* F1. The results show that the first three enzymes in the upper *tod* pathway has relaxed substrate specificities that accomodate the degradation of *n*-alkylbenzenes (C3-C7), styrene, cumene and biphenyl as well as the growth-supporting substrates. But the further degradation of the non-growth chemicals was blocked by TodF. This result is consistent with kinetic data obtained with purified TodF in which TodF has no detectable activity with 6-phenyl-HOHD and a negligible activity with 6-isopropyl-HOHD exhibiting 105-fold less activity compared with the growth substrate, 6-methyl-HOHD [15].

In addition, *n*-propylbenzene, styrene, cumene acted as inducers for the *tod* operon. For the inducer compounds to be growth substrates, TodF should be able to recognize their HOHD derivatives. In other report, 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 *todST* genes located at the end of the *tod* operon have been proposed to be involved in the transcriptional activation of the *tod* operon as two-component signal transduction pathway enzymes [12], the exact protein component for inducer binding has not been reported yet.

Acknowledgments: This work was supported by a research grant from the Korea Science and Engineering Foundation (KOSEF, 1999-211-00-001-1). I wish to thank Dr. David T. Gibson for providing *P. putida* F1 and *E. coli* JM109(pDTG601A) strains.

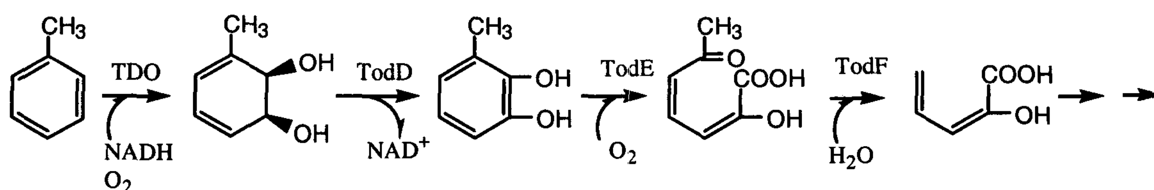

Fig. 1. The *tod* pathway in *P. putida* F1. Abbreviation for enzymes: TDO, toluene dioxygenase; TodD, *cis*-toluene dihydrodiol dehydrogenase; TodE, 3-methylcatechol dioxygenase; TodF, 6-methyl-HOHD hydrolase.

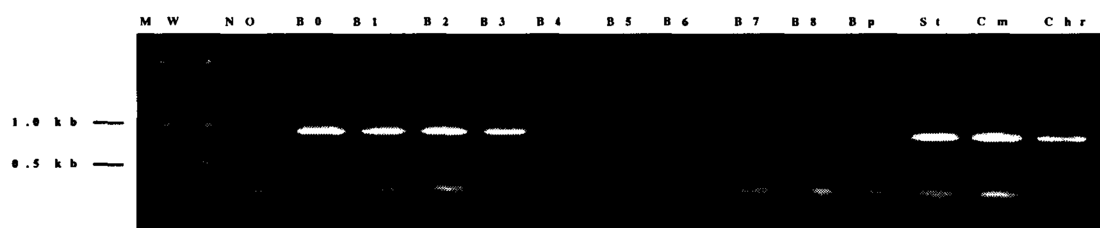

Fig. 2. RT-PCR amplification of *todF* mRNA transcripts purified from *P. putida* F1 grown in 5 mM succinate supplemented with each chemical on the abscissa. Abbreviations for chemicals: B0, benzene; B1, toluene; B2, ethylbenzene; B3, *n*-propylbenzene; B4, *n*-butylbenzene; B5, *n*-pentylbenzene; B6, *n*-hexylbenzene; B7, *n*-heptylbenzene; B8, *n*-octylbenzene; Bp, biphenyl; St, styrene; Cm, cumene. Chr represents a positive control in which *todF* gene was amplified by PCR from chromosomal DNA.

References

1 Applegate BM, SR Kehrmeyer and GS Sayler. 1998. A chromosomally based *tod-luxCDABE* whole-cell reporter for benzene, toluene, ethylbenzene, and xylene (BTEX) sensing. Appl Environ Microbiol 64: 2730-2735.

2 Boyd DR and GN Sheldrake. 1998. The dioxygenase-catalyzed formation of vicinal *cis*-diols. Natural Product Rep 15: 309-324.

3 Catelani D, A Colombi, C Sorlini and V Treccani. 1973. Metabolism of biphenyl. Biochem J 134: 1063-1066.

4 Eaton RW and KN Timmis. 1986. Characterization of a plasmid-specified pathway for catabolism of isopropylbenzene in *Pseudomonas putida* RE204. J Bacteriol 168: 123-131.

5 Finette B and DT Gibson. 1988. Initial studies on the regulation of toluene degradation *by Pseudomonas putida* F1. Biocatalysis 2: 29-37.

6 Furukawa K, J Hirose, A Suyama, T Zaiki and S Hayashida. 1993. Gene components responsible for discrete substrate specificity in the metabolism of biphenyl (*bph* operon) and toluene (*tod* operon). J Bacteriol 175: 5224-5232.

7 Gibson DT, B Gschwendt, WK Yeh and VM Kobal. 1973. Initial reactions in the oxidation of ethylbenzene by *Pseudomonas putida*. Biochemistry 12: 1520-1528.

8 Gibson DT, M Hensley, H Yoshioka, TJ Marbry. 1970. Formation of (+)-*cis*-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from toluene by *Pseudomonas putida*. Biochemistry 9: 1626-1630.

9 Gibson DT, GJ Zylstra and S. Chauhan. 1990. Biotransformations catalyzed by toluene dioxygenase from *Pseudomonas putida* F1. In: *Pseudomonas*: biotransformations, pathogenesis and evolving biotechnology (Silver S, AM Chakrabarty, B Iglewski and S Kaplan, ed), pp 121-132, American Society for Microbiology, Washington, DC.

10 Haigler BE, Pettigrew CA and JC Spain. 1992. Biodegradation of mixtures of substituted benzenes by *Pseudomonas* sp. strain JS150. Appl Environ Microbiol 58: 2237-2244.

11 Hudlicky T, D Gonzalez and DT Gibson. 1999. Enzymatic dihydroxylation of aromatics in enantioselective synthesis: expanding asymmetric methodology. Aldrichimica Acta 32: 35-62.

12 Lau PCK, Y Wang, A Patel, D Labbé, H Bergeron, R Brousseau, Y Konishi and M Rawlings. 1997. A bacterial basic region leucine zipper histidine kinase regulating toluene degradation. Proc Natl Acad Sci USA 94: 1453-1458.

13 Lau PCK, H Bergeron, D Labbé, Y Wang, R Brousseau and DT Gibson. 1994. Sequence and expression of the *todGIH* genes involved in the last three steps of toluene degradation by *Pseudomonas putida* F1. Gene 146: 7-13.

14 Mosqueda G, M-I Ramos-Gonzalalez, JL Ramos. 1999. Toluene metabolism by the solvent-tolerant *Pseudomonas putida* DOT-T1 strain, and its role in solvent impermeabilization. Gene 232: 69-76.

15 Seah SYK, G Terracina, JT Bolin, P Riebel, V. Snieckus and LD Elitis. 1998. Purification and preliminary characterization of a serine hydrolase involved in the microbial degradation of polychlorinated biphenyls. J Biol Chem 273: 22943-22949.

16 Smith MR and C Ratledge. 1989. Catabolism of alkylbenzenes by *Pseudomonas* sp. NCIB 10643. Appl Microbiol Biotech 32: 68-75.

17 Warhurst AM, KF Clarke, RA Hill, RA Holt and CA Fewson. 1994. Metabolism of styrene by *Rhodococcus rhodochrous* NCIMB 13259. Appl Environ Microbiol 60: 1137-1145.

18 Zylstra GJ and DT Gibson. 1989. Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. J Biol Chem 264: 14940-14946.