Biocatalysis and Biotransformation, 2004 VOL. 22 (4). pp. 283–289

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Physiological Relevance of Successive Hydroxylations of Toluene by Toluene *para*-Monooxygenase of *Ralstonia pickettii* PKO1

AYELET FISHMAN, YING TAO and THOMAS K. WOOD*

Departments of Chemical Engineering and Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269-3222

(Received for publication 20 July 2004; Revised received 7 September 2004)

We have recently found that toluene *para*-monooxygenase (TpMO) of *Ralstonia pickettii* PKO1 (encoded by *tbuA1UBVA2C*) performs successive hydroxylations of benzene (*Appl. Environ. Microbiol.* 70: 3814, 2004) as well as hydroxylates toluene to a mixture of 90% *p*-cresol and 10% *m*-cresol which are then further oxidized to 100% 4-methylcatechol (*J. Bacteriol.* 186: 3117, 2004) whereas it was thought previously that TpMO forms 100% *m*-cresol and is not capable of successive hydroxylations. Here we propose a modification of the degradation pathway originally described by Olsen *et al*. (*J. Bacteriol*. 176: 3749, 1994) that now relies primarily on TpMO for conversion of toluene to 4-methylcatechol (instead of *m*-cresol) since both *m*-cresol and *p*-cresol are shown here to be good substrates for *Escherichia coli* expressing TpMO ($V_{max}/K_m = 0.046$, 0.036, and 0.055 mL min^{-1} mg^{-1} protein for the oxidation of toluene, *m*-cresol, and *p*-cresol, respectively). In light of the broader activity of TpMO, phenol hydroxylase (encoded by *tbuD*) appears to facilitate conversion of any *m*-cresol or *p*-cresol formed from toluene oxidation by TpMO to 4-methylcatechol; hence, the cell has a redundant method for making this important intermediate 4-methylcatechol. Further, it is suggested that the physiological relevance of the 10% *m*-cresol formed from toluene oxidation by TpMO is needed for induction of the *meta* cleavage operon *tbuWEFGKIHJ* to enable full metabolism of toluene since *p*-cresol (and *o*-cresol) do not induce the *meta*-cleavage pathway. Therefore both the successive hydroxylation of toluene by TpMO and the product distribution are of physiological relevance to the cell.

Keywords: Phenol hydroxylase; Toluene *para*-monooxygenase Toluene pathway

INTRODUCTION

Toluene monooxygenases are multi-component non-heme, di-iron enzymes capable of hydroxylating aromatic molecules using molecular oxygen and NADH as a co-factor. They are invaluable biocatalysts due to their high regio- and stereoselectivity and their ability to facilitate reactions with chemically stable starting compounds (Burton, 2003; van Beilen *et al*., 2003). Toluene monooxygenases have been shown to be useful catalysts for bioremediation (Canada *et al*., 2002; Rui *et al*., 2004a; Shim *et al*., 2001) and for biocatalysis (Fishman *et al*., 2004a; Schmid *et al*., 2001; Tao *et al*., 2004b; Vardar and Wood, 2004).

Toluene *para*-monooxygenase (TpMO) of *Ralstonia pickettii* PKO1, formerly known as toluene 3-monooxygenase (T3MO), was recently shown by us to both perform hydroxylation of monosubstituted benzenes primarily at the *para* position (Fishman *et al*., 2004b) and to successively oxidize benzene to phenol, catechol, and 1,2,3-trihydroxybenzene (Tao *et al*., 2004a). Thus, this enzyme produces 90% *p*-cresol and 10% *m*-cresol from toluene which are both subsequently oxidized to 4-methylcatechol (Fishman *et al*., 2004b). There is thorough and well-documented literature on the toluene degradation pathway of TpMO and on the role of the various genes in the *tbu* operon encoding this pathway (Byrne *et al*., 1995; Byrne and Olsen, 1996; Kahng *et al*., 2000; Kukor and Olsen, 1990, 1991, 1992; Leahy *et al*., 1997; Olsen *et al*., 1994). Originally, toluene was thought to be oxidized initially to *m*-cresol by TpMO which was then oxidized by phenol hydroxylase to 3-methylcatechol with subsequent *meta* cleavage of the aromatic ring by catechol 2,3-dioxygenase (Olsen *et al*., 1994). In this paper we report that the newly discovered double hydroxylation of toluene to 4-methylcatechol by TpMO as

* Corresponding author. Tel.: (860) 486-2483. Fax: (860) 486-2959. Email twood@engr.uconn.edu.

ISSN 1024-2422 print/ISSN 1029-2446 online
DOI: 10.1080/10242420400012008

well as the regiospecificity of the reaction product mixture are physiologically relevant. It is also argued that the phenol hydroxylase (TbuD, a flavin monooxygenase (Kukor and Olsen, 1992)) is redundant for 4-methylcatechol production from *m*-cresol and *p*-cresol.

MATERIALS AND METHODS

Chemicals

Toluene and *p*-cresol (99 + %) were obtained from Acros Organics (Morris Plains, NJ). *o*-Cresol (99 + %), *m*-cresol (97%), 3-methylcatechol, and 4-methylcatechol were obtained from Sigma-Aldrich Co. (Milwaukee, WI). All materials used were of the highest purity available and were used without further purification.

Bacterial Strains and Growth Conditions

To stably and constitutively express TpMO in *Escherichia coli*, the expression vector pBS(Kan)TpMO was constructed as described earlier (Tao *et al*., 2004a). *E. coli* TG1 (*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′ [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) with pBS(Kan)TpMO was routinely cultivated at 37°C with shaking at 250 rpm on a C25 incubator shaker (New Brunswick Scientific Co., Edison, NJ) in Luria-Bertani (LB) medium (Sambrook *et al*., 1989) supplemented with kanamycin at 100 μg mL^{-1} to maintain the plasmids. All experiments were conducted by diluting overnight cells to an optical density at 600 nm (OD) of 0.1 to 0.2 and growing to an OD of 1.2. The exponentially-grown cells were centrifuged at 13,000 × *g* for 8 min at 25°C in a J2-HS centrifuge (Beckman Coulter, Inc., Palo Alto, CA) and resuspended in Tris-HNO_3 buffer (50 mM, pH 7.0). Expression of wild-type TpMO from pBS(Kan)TpMO within *E. coli* strains (henceforth TG1(TpMO)) produced blue-colored cells on agar plates and in broth cultures. The blue color is indicative of indigo, formed by oxidation of indole from tryptophan (Rui *et al*., 2004b).

Enzymatic Activity

The rates of oxidation of *m*-cresol and *p*-cresol were determined using high-pressure liquid chromatography (HPLC) with a Zorbax SB-C8 column (Agilent Technologies, Palo Alta, CA, 5 μm, 4.6 × 250 mm) as described earlier (Fishman *et al*., 2004b). Toluene oxidation was performed using a Hewlett-Packard 6890N gas chromatograph (GC) equipped with an EC-WAX capillary column (30 m × 0.25 mm, 0.25 μm thickness; Alltech Associates, Inc., Deerfield, IL) and a flame ionization detector as described earlier (Fishman *et al*., 2004b).

RESULTS

Much work has been performed in the last decade on the toluene degradation pathway located on the chromosome of *R. pickettii* PKO1 (Byrne *et al*., 1995; Byrne and Olsen, 1996; Kahng *et al*., 2000; Kukor and Olsen, 1990, 1991, 1992; Leahy *et al*., 1997; Olsen *et al*., 1994). The enzymes in the pathway are divided into three separate operons: *tbuA1UBVA2CT* encoding TpMO and the transcriptional activator TbuT, *tbuD* encoding phenol hydroxylase, and *tbuWEFGKIHJ* encoding enzymes of the *meta*-cleavage pathway for conversion of catechol and methylcatechols to tricarboxylic acid cycle intermediates (Kahng *et al*., 2000; Olsen *et al*., 1997). Originally, these operons were thought to be regulated by three separate regulators, TbuT for TpMO transcription, and TbuR and TbuS for *tbuD* and *tbuWEFGKIHJ*, respectively (Kukor and Olsen, 1991, 1992; Olsen *et al*., 1994); however, later publications indicate that TbuT regulates all three operons via three different promoters (Byrne and Olsen, 1996; Kahng *et al*., 2000; Olsen *et al*., 1997). Kahng *et al*. (2000) recently described the role of TbuX, another protein in the *tbu* regulon which is also regulated by TbuT, as an outer membrane protein responsible for transport of toluene into the cell. A summary of the inducing compounds, non-inducing compounds, and substrates for the three main enzymatic steps in the toluene degradation pathway are presented in Table I. The role of phenol as an inducer for TpMO is unclear in the literature. Early work indicates phenol is not an inducer for TpMO (Leahy *et al*., 1997; Olsen *et al*., 1994) whereas later works show it is an inducer, albeit a poor one compared with toluene and benzene (Kahng *et al*., 2000). Therefore phenol is listed both as an inducer and a non-inducer in Table I. Our goal was to see whether the newly published results regarding the oxidation of toluene to 90% *p*-cresol and 10% *m*-cresol (Fishman *et al*., 2004b) fit with the pathway described earlier for allegedly 100% *m*-cresol formation from toluene.

Olsen *et al*. proposed that *m*-cresol formed from toluene by TpMO is converted to 3- and 4-methylcatechol by phenol hydroxylase (Kukor and Olsen, 1992; Olsen *et al*., 1994). Our results indicate that TpMO oxidizes toluene at an apparent V_{max} of 11.5 ± 0.3 nmol min^{-1} mg^{-1} protein (Fishman *et al*., 2004b), and almost as rapidly forms 4-methylcatechol from *p*-cresol and *m*-cresol at apparent V_{max} values of 5.5 ± 0.32 and 3.6 ± 0.2 nmol min^{-1} mg^{-1} protein, respectively (Table II). The similarity in the V_{max}/K_m values (Table II) of the three reactions indicate comparable efficiencies of the enzyme for the two sequential hydroxylations.

Toluene is not a substrate for phenol hydroxylase of *R. pickettii* PKO1 in the metabolic pathway since

TABLE I Inducing compounds and substrates for the three main enzymatic steps in the toluene degradation pathway of *R. pickettii* PKO1.

	TpMO	Phenol hydroxylase	*meta*-Cleavage enzymes
Genes	*tbuA1UBVA2C*	*tbuD*	*tbuWEFGKIHJ*
Regulator	TbuT[4]	TbuT[9]	TbuT[9]
Promoter	*ptbuA1*[4,13]	*ptbuD*[9]	*ptbuW*[11]
Inducers	toluene,[1,3,4] benzene,[1,3,4] ethylbenzene,[1,4] *m*-cresol,[1,3,4] chlorobenzene,[3] *o*-cresol,[3,4] trichloroethylene,[4,8] phenol[4,10]	phenol,[6,9] *m*-cresol[6] toluene[9]	phenol,[5] *m*-cresol[5] toluene was not tested
Non-inducers	*o*-xylene[1,4], *m*-xylene[1,4], *p*-xylene,[1,4] *p*-cresol,[1,3,4] phenol,[1,3] styrene,[1] phenylacetylene,[1] benzyl alcohol,[4] benzaldehyde[4]	*o*-cresol,[6] *p*-cresol,[6] catechol,[6] 3-methylcatechol,[6] 4-methylcatechol[6]	*o*-cresol,[5] *p*-cresol,[5] catechol,[5] 4-methylcatechol[5]
Substrates	toluene,[1] benzene,[1] ethylbenzene,[1] *o*-xylene, [1] *m*-xylene, [1] *p*-xylene,[1] styrene,[1] phenylacetylene,[1] phenol,[2] *m*-cresol[12], *p*-cresol,[12] catechol[2]	phenol,[6,7] *o*-cresol,[6,7] *m*-cresol,[6,7] *p*-cresol,[6,7] catechol,[6,7] resorcinol,[6,7] chlorophenols,[6,7] fluorophenols,[7] aminophenols[7]	catechol,[5] 3-methylcatechol,[5] 4-methylcatechol[5]

[1]ref. (Olsen *et al*., 1994); [2]ref. (Tao *et al*., 2004a); [3]ref. (Leahy *et al*., 1997); [4]ref. (Byrne and Olsen, 1996); [5]ref. (Kukor and Olsen, 1991); [6]ref. (Kukor and Olsen, 1990); [7]ref. (Kukor and Olsen, 1992); [8]ref. (Leahy *et al*., 1996); [9]ref. (Olsen *et al*., 1997); [10]ref. (Kahng *et al*., 2001); [11]ref. (Kukor and Olsen, 1996); [12]this work; [13]also the promoter for *tbuT* which is located downstream of *tbuA1UBVA2C*[4].

deleting TpMO from pRO1957 (encoding the entire pathway) resulted in loss of ability to grow on toluene (Olsen *et al*., 1994); therefore, phenol hydroxylase cannot initiate the reaction with toluene. Since TpMO can efficiently oxidize toluene to 4-methyl catechol, the substrate for catechol 2,3-dioxygenase (TbuE), it seems that phenol hydroxylase is redundant for toluene metabolism in *R. pickettii* PKO1 rather than necessary for 3- and 4-methylcatechol formation (Fig. 1). The fact that TbuE (the first enzyme in the *meta*-cleavage operon) is induced by *m*-cresol but not by *p*-cresol or 4-methylcatechol (Table I), may explain the product distribution of toluene oxidation by TpMO. It is hypothesized that the small but substantial amount of *m*-cresol formed is needed to induce the activity of TbuE and the entire *tbuWEFGKIHJ* operon to enable full degradation of toluene to propionic aldehyde and pyruvate. The *m*-cresol will also induce phenol hydroxylase (Table I) that can contribute in the hydroxylation of *m*- and *p*-cresol to 3- and 4-methylcatechol. The major product of our proposed pathway, 4-methylcatechol, is recognized by TbuE of *P. pickettii* PKO1 (Kukor and Olsen, 1991) just as well as the 3-methylcatechol that was originally proposed as the product of toluene oxidation (Olsen *et al*., 1994), so the proposed 4-methylcatechol intermediate fits well into the *meta*-ring cleavage pathway described earlier. Importantly, the 2-hydroxy-5-methyl-*cis*,*cis*-muconic semialdehyde, product of 4-methylcatechol oxidation by TbuE, has also been shown to be a substrate of the *tbuWEFGKIHJ meta*-cleavage operon since *P. aeruginosa* cells with plasmid pRO1996 containing *tbuWEFGKIHJ* utilize 4-methylcatechol as a carbon source (Kukor and Olsen, 1991). So all of the downstream intermediates of TpMO oxidation of toluene have been shown to be pathway intermediates for the utilization of toluene for carbon and energy. The revised pathway for utilization of toluene by *R. pickettii* PKO1 is presented in Fig. 1.

It appears that phenol hydroxylase is completely redundant in the toluene degradation pathway of *R. pickettii* PKO1 and its main role is in helping TpMO to transform cresols to methylcatechols and not to enable the cell to grow on phenol as suggested earlier. Phenol supports growth of *P. aeruginosa* PAO1 harboring pRO1957 (encoding the entire pathway) (Olsen *et al*., 1994) but is a poor inducer for both TpMO (Byrne and Olsen, 1996; Kahng *et al*., 2001; Olsen *et al*., 1994) and phenol hydroxylase

TABLE II Saturation kinetic values (apparent V_{max} and K_m) and product distribution for toluene, *m*-cresol, and *p*-cresol oxidation by *E. coli* TG1 cells expressing TpMO from pBS(Kan)TpMO.

	Kinetics of oxidation			Regiospecificity of the oxidation reaction		
Reaction	Apparent V_{max}, nmol min^{-1} mg^{-1} protein	Apparent K_m, μM	V_{max}/K_m, ml min^{-1} mg^{-1} protein	*p*-cresol, %	*m*-cresol, %	4-methylcatechol, %
toluene → cresol	11.5 ± 0.3	250	0.046	90	10	N/A
p-cresol → 4-methylcatechol[a]	5.5 ± 0.3	100	0.055	N/A[b]	N/A	100
m-cresol → 4-methylcatechol[a]	3.6 ± 0.2	100	0.036	N/A	N/A	100

[a]Based on HPLC analysis for a period of 30 min with substrate concentrations of 25, 50, 125, 250, 500, 800, and 1000 μM.
[b]N/A: not applicable.

(Olsen *et al*., 1997) indicating the metabolic pathway for phenol oxidation can start with either enzyme. Both TpMO and phenol hydroxylase are induced by phenol and can utilize phenol efficiently (Table I) and therefore the cell can grow on phenol without the presence of phenol hydroxylase. Phenol is also an inducer for catechol 2,3-dioxygenase providing the ability for complete catabolism of phenol to pyruvate and acetaldehyde.

DISCUSSION

In this brief report we have shown that the sequential hydroxylation of toluene by TpMO to 90% *p*-cresol and 10% *m*-cresol followed by 100% formation of 4-methylcatechol is of physiological relevance to *R. pickettii* PKO1. 4-Methylcatechol can be fully utilized by the *meta* cleavage pathway described earlier by Kukor and Olsen (1991). Double hydroxylation of toluene to methylcatechol has been reported previously for other combinations of a soluble diiron monooxygenases and phenol hydroxylase (primarily *ortho*-hydroxylating enzymes). *Burkholderia* sp. strain JS150 contains two plasmid-derived monooxygenases encoded by the *tbc1* and *tbc2* operons (Kahng *et al*., 2001). The *ortho*-hydroxylating Tbc2 monooxygenase converts toluene to *o*-cresol and further to 3-methylcatechol and is highly similar to TpMO of *R. pickettii* PKO1 ($>92\%$ identity) whereas Tbc1 was only able to convert *o*-cresol to 3-methylcatechol and was genetically related to multiple-component phenol hydroxylases (Kahng *et al*., 2001). This *tbc*-encoded pathway is organized similarly to the toluene degradation pathway in *R. pickettii* PKO1 with a contiguous toluene monooxygenase and phenol hydroxylase. Another plasmid-encoded toluene/benzene 2-monooxygenase (Tb2m) was isolated from *Burkholderia* sp. JS150 and was shown to transform toluene to *o*-cresol and further to 3-methylcatechol (Johnson and Olsen, 1995). Tb2m is phylogenetically related to phenol hydroxylases Tbc1 of *Burkholderia* sp. JS150 and toluene *ortho*-monooxygenase (TOM) of *B. cepacia* G4. The *tbm* regulon of *Burkholderia* sp. JS150 also includes a *para*-hydroxylating toluene 4-monooxygenase (Tb4m) that can hydroxylate only unactivated benzene nuclei but cannot hydroxylate cresols (Johnson and Olsen, 1997). This pathway differs from that of Fig. 1 in that both monooxygenases of the *tbm* regulon initiate oxidation of toluene whereas only TpMO initiates degradation in *R. pickettii* PKO1. Both pathways have one regulating enzyme for both monooxygenases (TbmR in the *tbm* operon and TbuT in the *tbu* operon of *R. pickettii* PKO1).

Another well-studied monooxygenase capable of double hydroxylation of toluene is toluene/*o*-xylene monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1. ToMO has been shown to convert toluene to a mixture of *o*-, *m*-, and *p*-cresol with subsequent hydroxylation to 3- and 4-methylcatechol (Bertoni *et al*., 1996, 1998) and is unique in that it displays a broad substrate range along with a very relaxed regiospecificity unlike other toluene monooxygenases (Bertoni *et al*., 1998). The 3- and 4-methylcatechols are further catabolized by a *meta* cleavage pathway similar to the *R. pickettii* PKO1 pathway we propose here (Bertoni *et al*., 1996). The ToMO operon *touABCDEF* is induced primarily by phenolic compounds (*o*-, *m*-, *p*-cresol, and various dimethyl phenols) through TouR (Arenghi *et al*., 1999), whereas the TpMO *tbuA1UBVA2C* is regulated primarily through substrate induction (toluene and benzene) (Byrne and Olsen, 1996). Interestingly, toluene and *o*-xylene catabolism are organized in two operons, one encoding for ToMO and the other encoding a multi-component phenol hydroxylase (PH), catechol 2,3-dioxygenase, and the remaining *meta*-cleavage pathway enzymes (Arenghi *et al*., 2001; Cafaro *et al*., 2004). Both operons are regulated by TouR similarly to TbuT in *R. pickettii* PKO1. A recent study by Cafaro *et al*. showed that both ToMO and PH of *P. stutzeri* OX1 could oxidize benzene to phenol and phenol to catechol, albeit at different efficiencies (Cafaro *et al*., 2004). The K_{cat}/K_m for benzene oxidation was 1810 and 3.7 ($10^3\ s^{-1}\ \mu M^{-1}$) for ToMO and PH respectively, and 460 and 1696 for phenol oxidation, suggesting that the PH functions mainly to drain the monohydroxylated compounds and prevent them for accumulating within the cell (Cafaro *et al*., 2004).

It is therefore evident from the present study as well as from previous work, that *Pseudomonas* strains may have multiple upper pathways to oxidize substituted benzenes. Previously, these pathways included a toluene 2-monooxygenase and a phenol hydroxylase, and the reactions catalyzed by the phenol hydroxylase appear redundant with respect to cresol conversion to substituted catechols; we have shown here that a similar genetic arrangement exists for a *para*-hydroxylating enzyme. Since T4MO (toluene 4-monooxygenase from *P. mendocina* KR1) has also been shown by us to perform double hydroxylations of benzene and toluene with substantial rates (Tao *et al*., 2004a,b), perhaps the proposed pathway involving *p*-cresol conversion to protocatechuate prior to *meta* cleavage (Whited and Gibson, 1991) may be more complex than originally thought.

One unique feature of the *R. pickettii* PKO1 pathway compared to that of *Burkholderia* sp. JS150 and *P. stutzeri* OX1 described above, is the structure of its phenol hydroxylase. TbuD is a simple flavoprotein monooxygenase translated from a single gene and is

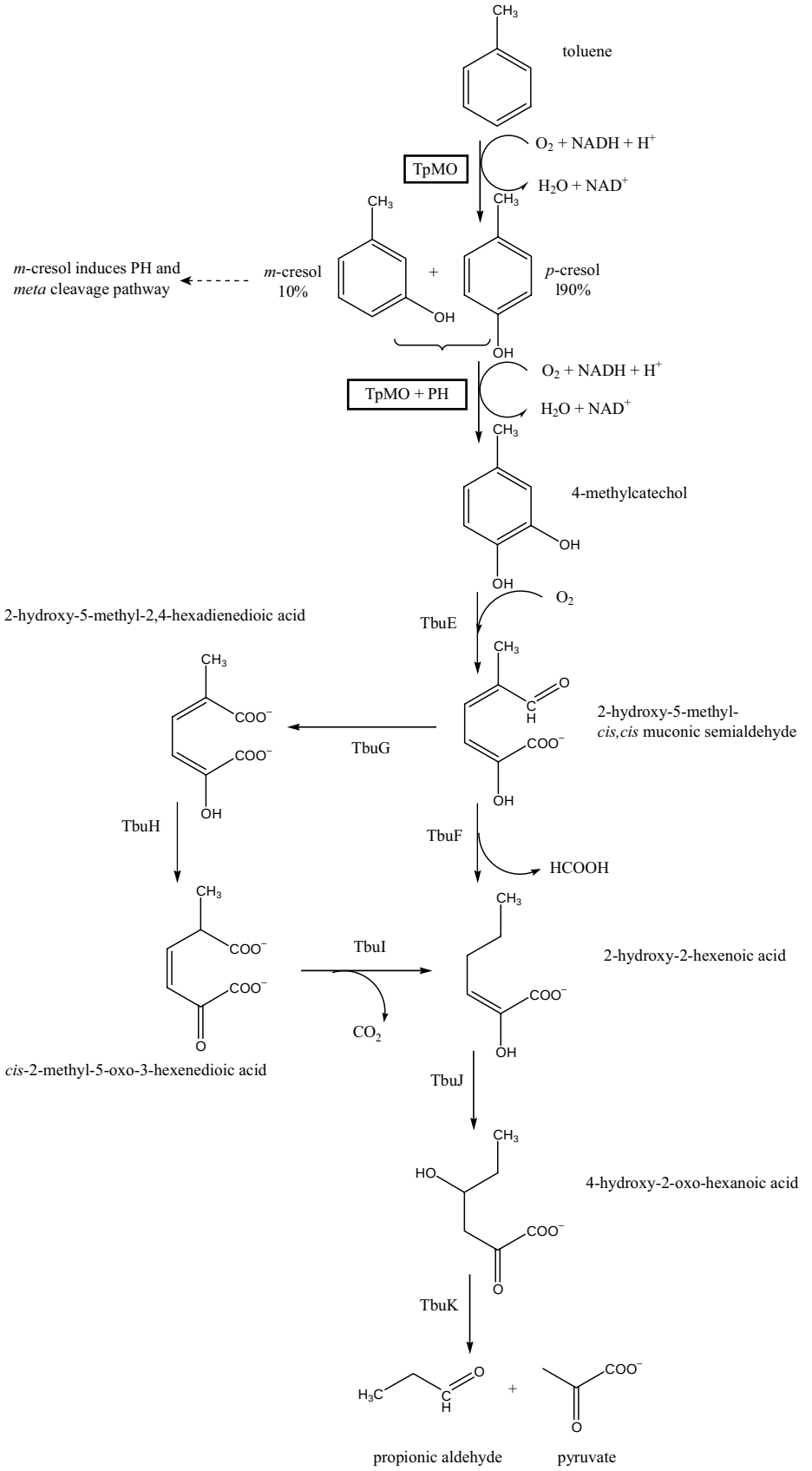

FIGURE 1 Proposed modification of the pathway for degradation of toluene by *R. pickettii* PKO1. TpMO, toluene *para*-monooxygenase; PH, phenol hydroxylase; TbuE, catechol-2,3-dioxygenase; TbuF, 2-hydroxymuconate semialdehyde hydrolase; TbuG, 2-hydroxymuconate semialdehyde dehydrogenase; TbuH, 4-oxalocrotonate isomerase; TbuI, 4-oxalocrotonate decarboxylase; TbuJ, 2-hydroxypent-2,4-dienoate hydratase; and TbuK, 4-hydroxy-2-oxovalerate aldolase.

not a multi-component enzyme (Kukor and Olsen, 1992; Olsen *et al*., 1997). However, the similarity of adjacent *crpABCD* genes (these genes are downstream of the *ptbuD* promoter but are not translated into peptides) with *tb2m* of *Burkholderia* sp. JS150 suggests that these two strains may have evolved from a progenitor strain (Notomista *et al*., 2003; Olsen *et al*., 1997).

The existence of multiple monooxygenases in one strain may provide the cell with a competitive advantage and with the ability to utilize a large range of chemically different substrates effectively. The phenol hydroxylase of *R. pickettii* PKO1 is redundant with respect to hydroxylation of *m*-cresol and *p*-cresol to 4-methylcatechol, as this reaction was shown to be performed effectively by TpMO with comparable V_{max}/K_m values as toluene oxidation. It is also redundant with respect to hydroxylation of phenol as this reaction was also shown to be performed efficiently by TpMO (Tao *et al*., 2004a).

It appears that the *m*-cresol formed from toluene oxidation is needed for induction of the redundant phenol hydroxylase and the lower degradation pathway comprising of the *meta*-cleavage enzymes to enable full utilization of toluene as a carbon source since *o*-cresol and *p*-cresol are not inducers of these two operons. Therefore, the double hydroxylation of toluene by TpMO and its somewhat relaxed regiospecificity are of physiological relevance to *R. pickettii* PKO1. Purely *para*-hydroxylating enzymes are possible as we have constructed one with reasonable activity from T4MO (TmoA variant G103S/A107T) (Tao *et al*., 2004b); hence, the cell must choose to make some *m*-cresol as a result of the regulatory machinery.

Acknowledgements

This research was supported by the National Science Foundation (BES-0124401) and the U.S. Environmental Protection Agency.

References

Arenghi, F.L.G., Berlanda, D., Galli, E., Sello, G. and Barbieri, P. (2001) Organization and regulation of *meta* cleavage pathway genes for toluene and *o*-xylene derivative degradation in *Pseudomonas stutzeri* OX1, *Appl. Environ. Microbiol.* **67**, 3304–3308.

Arenghi, F.L.G., Pinti, M., Galli, E. and Barbieri, P. (1999) Identification of the *Pseudomonas stutzeri* OX1 toluene-*o*-xylene monooxygenase regulatory gene (*touR*) and its cognate promoter, *Appl. Environ. Microbiol.* **65**, 4057–4063.

Bertoni, G., Bolognese, F., Galli, E. and Barbieri, P. (1996) Cloning of the genes for and characterization of the early stages of toluene and *o*-xylene catabolism in *Pseudomonas stutzeri* OX1, *Appl. Environ. Microbiol.* **62**, 3704–3711.

Bertoni, G., Martino, M., Galli, E. and Barbieri, P. (1998) Analysis of the gene cluster encoding toluene/*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1, *Appl. Environ. Microbiol.* **64**, 3626–3632.

Burton, S.G. (2003) Oxidizing enzymes as biocatalysts, *Trends Biotech.* **21**, 543–549.

Byrne, A.M., Kukor, J.J. and Olsen, R.H. (1995) Sequence analysis of the gene cluster encoding toluene-3-monooxygenase from *Pseudomonas pickettii* PK01, *Gene* **154**, 65–70.

Byrne, A.M. and Olsen, R.H. (1996) Cascade regulation of the toluene-3-monooxygenase operon (*tbuA1UBVA2C*) of *Burkholderia pickettii* PKO1: Role of the *tbuA1* promoter (*PtbuA1*) in the expression of its cognate activator, TbuT, *J. Bacteriol.* **178**, 6327–6337.

Cafaro, V., Izzo, V., Scognamiglio, R., Notomista, E., Capasso, P., Casbarra, A., Pucci, P. and Di Donato, A. (2004) Phenol hydroxylase and toluene/*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1: interplay between two enzymes, *Appl. Environ. Microbiol.* **70**, 2211–2219.

Canada, K.A., Iwashita, S., Shim, H. and Wood, T.K. (2002) Directed evolution of toluene *ortho*-monooxygenase for enhanced 1-naphthol synthesis and chlorinated ethene degradation, *J. Bacteriol.* **184**, 344–349.

Fishman, A., Tao, Y., Bentley, W.E. and Wood, T.K. (2004a) Protein engineering of toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 for synthesizing 4-nitrocatechol from nitrobenzene, *Biotechnol. Bioeng.* **87**, 779–790.

Fishman, A., Tao, Y. and Wood, T.K. (2004b) Toluene 3-mono oxygenase of *Ralstonia pickettii* PKO1 is a para-hydroxylating enzyme, *J. Bacteriol.* **186**, 3117–3123.

Johnson, G.R. and Olsen, R.H. (1995) Nucleotide sequence analysis of genes encoding a toluene/benzene-2-monooxygenase from *Pseudomonas* sp. strain JS150, *Appl. Environ. Microbiol.* **61**, 3336–3346.

Johnson, G.R. and Olsen, R.H. (1997) Multiple pathways for toluene degadation in *Burkholderia* sp. strain JS150, *Appl. Environ. Microbiol.* **63**, 4047–4052.

Kahng, H.-Y., Byrne, A.M., Olsen, R.H. and Kukor, J.J. (2000) Characterization and role of *tbuX* in utilization of toluene by *Ralstonia pickettii* PKO1, *J. Bacteriol.* **182**, 1232–1242.

Kahng, H.-Y., Malinverni, J.C., Majko, M.M. and Kukor, J.J. (2001) Genetic and functional analysis of the tbc operons for catabolism of alkyl- and chloroaromatic compounds in *Burkholderia sp.* strain JS150, *Appl. Environ. Microbiol.* **67**, 4805–4816.

Kukor, J.J. and Olsen, R.H. (1990) Molecular cloning, characterization, and regulation of a *Pseudomonas pickettii* PKO1 gene encoding phenol hydroxylase and expression of the gene in *Pseudomonas aeruginosa* PAO1c, *J. Bacteriol.* **172**, 4624–4630.

Kukor, J.J. and Olsen, R.H. (1991) Genetic organization and regulation of a *meta* cleavage pathway for catechols produced from catabolism of toluene, benzene, phenol, and cresols by *Pseudomonas pickettii* PKO1, *J. Bacteriol.* **173**, 4587–4594.

Kukor, J.J. and Olsen, R.H. (1992) Complete nucleotide sequence of *tbuD*, the gene encoding phenol/cresol hydroxylase from *Pseudomonas pickettii* PKO1, and functional analysis of the encoded enzyme, *J. Bacteriol.* **174**, 6518–6526.

Kukor, J.J. and Olsen, R.H. (1996) Catechol 2,3-dioxygenases functional in oxygen-limited (hypoxic) environments, *Appl. Environ. Microbiol.* **62**, 1728–1740.

Leahy, J.G., Byrne, A.M. and Olsen, R.H. (1996) Comparison of factors influencing trichloroethylene degradation by toluene-oxidizing bacteria, *Appl. Environ. Microbiol.* **62**, 825–833.

Leahy, J.G., Johnson, G.R. and Olsen, R.H. (1997) Cross-regulation of toluene monooxygenases by the transcriptional activators TbmR and TbuT, *Appl. Environ. Microbiol.* **63**, 3736–3739.

Notomista, E., Lahm, A., Di Donato, A. and Tramontano, A. (2003) Evolution of bacterial and archaeal multicomponent monooxygenases, *J. Mol. Evol.* **56**, 435–445.

Olsen, R.H., Kukor, J.J., Byrne, A.M. and Johnson, G.R. (1997) Evidence for the evolution of a single component phenol/cresol hydroxylase from a multicomponent toluene monooxygenase, *J. Industr. Micro. Biotech.* **19**, 360–368.

Olsen, R.H., Kukor, J.J. and Kaphammer, B. (1994) A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1, *J. Bacteriol.* **176**, 3749–3756.

Rui, L., Kwon, Y., Fishman, A., Reardon, K.F. and Wood, T.K. (2004a) Saturation mutagenesis of toluene *ortho*-monooxygenase of *Burkholderia cepacia* G4 for enhanced 1-naphthol

synthesis and chloroform degradation, *Appl. Environ. Microbiol.* **70**, 3246–3252.

Rui, L., Reardon, K.F. and Wood, T.K. (2004b) Protein engineering of toluene *ortho*-monooxygenase of *Burkholderia cepacia* G4 for regiospecific hydroxylation of indole to form indigoid compounds. *Appl. Microbiol. Biotechnol.*, in press.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning, a laboratory manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Schmid, A., Dordick, J.S., Hauer, B., Kiener, A., Wubbolts, M. and Witholt, B. (2001) Industrial biocatalysis today and tomorrow, *Nature* **409**, 258–268.

Shim, H., Ryoo, D., Barbieri, P. and Wood, T.K. (2001) Aerobic degradation of mixtures of tetrachloroethylene, trichloroethylene, dichloroethtylenes, and vinyl chloride by toluene-*o*-xylene monooxygenase of *Pseudomonas stutzeri* OX1, *Appl. Microbiol. Biotechnol.* **56**, 265–269.

Tao, Y., Fishman, A., Bentley, W.E. and Wood, T.K. (2004a) Oxidation of benzene to phenol, catechol, and 1,2,3-trihydroxybenzene by toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 and toluene 3-monooxygenase of *Ralstonia pickettii* PKO1, *Appl. Environ. Microbiol.* **70**, 3814–3820.

Tao, Y., Fishman, A., Bentley, W.E. and Wood, T.K. (2004b) Saturation mutagenesis of toluene 4-monooxygenase from *Pseudomonas mendocina* KR1 for the production of 3-methoxy catechol, methoxyhydroquinone, and methylhydroquinone, *J. Bacteriol.* **186**, 4705–4713.

van Beilen, J.B., Duetz, W.A., Schmid, A. and Witholt, B. (2003) Practical issues in the application of oxygenases, *Trends Biotech.* **21**, 170–177.

Vardar, G. and Wood, T.K. (2004) Protein engineering of toluene-*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1 for synthesizing of 4-methylresorcinol, methyhydroquinone, and pyrogallol, *Appl. Environ. Microbiol.* **70**, 3253–3262.

Whited, G.M. and Gibson, D.T. (1991) Separation and partial characterization of the enzymes of the toluene-4-monooxygenase catabolic pathway in *Pseudomonas mendocina* KR1, *J. Bacteriol.* **173**, 3017–3020.