REVIEW

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# Bacterial Aerobic Degradation of Benzene, Toluene, Ethylbenzene and Xylene

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**ABSTRACT.** Several aerobic metabolic pathways for the degradation of benzene, toluene, ethylbenzene and xylene (BTEX), which are provided by two enzymic systems (dioxygenases and monooxygenases), have been identified. The monooxygenase attacks methyl or ethyl substituents of the aromatic ring, which are subsequently transformed by several oxidations to corresponding substituted pyrocatechols or phenylglyoxal, respectively. Alternatively, one oxygen atom may be first incorporated into aromatic ring while the second atom of the oxygen molecule is used for oxidation of either aromatic ring or a methyl group to corresponding pyrocatechols or protocatechuic acid, respectively. The dioxygenase attacks aromatic ring with the formation of 2-hydroxy-substituted compounds. Intermediates of the "upper" pathway are then mineralized by either *ortho*- or *meta*-ring cleavage ("lower" pathway). BTEX are relatively water-soluble and therefore they are often mineralized by indigenous microflora. Therefore, natural attenuation may be considered as a suitable way for the clean-up of BTEX contaminants from gasoline-contaminated soil and groundwater.

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## 1 INTRODUCTION

Benzene, toluene, ethylbenzene and xylene (BTEX) are volatile simple aromatic hydrocarbons commonly present in crude petroleum and petroleum products such as gasoline. They are also produced in Tg (*i.e.* megatons) per year as bulk chemicals for industrial use as solvents and starting materials for the manufacture of pesticides, plastics and synthetic fibers (Harwood *et al.* 1997). BTEX are considered to be one of the major causes of environmental pollution because of widespread occurrence of leakage from underground petroleum storage tanks and spills at petroleum production wells, refineries, pipelines and distribution terminals (Fries *et al.* 1994). Contamination of groundwater with the BTEX compounds is difficult to remedy because these compounds are relatively water-insoluble (Table I) and can diffuse rapidly once introduced into aquifers. Techniques for *in situ* bioremediation are used to eliminate or reduce contamination levels in aquifers (*see, e.g.*, Damborský *et al.* 2000; Obuekwe *et al.* 2001).

BTEX compounds are included in European priority lists and are frequently observed as groundwater pollutants due to their widespread use in petroleum industry or as solvents. Despite the toxicity and persistence, many microorganisms are able to transform or mineralize these compounds and use them as a sole source of carbon and energy (Damborský *et al.* 2000).

Mineralization of BTEX has been reported under aerobic conditions as well as in the presence of other electron acceptors. However, diverse pathways are assumed to be responsible for a productive breakdown of BTEX components under aerobic conditions. Therefore, complete BTEX mineralization involves

a complex community of microorganisms including those responsible for the degradation of misrouted substrates that have not been characterized until present.

**Table I.** Water solubility ($w_{vaq}$, %) and octanol–water partition coefficients ($K_{ow}$) of BTEX

| Compound | Benzene | Toluene | Ethylbenzene | *o*-Xylene | *m*-Xylene | *p*-Xylene |
|---|---|---|---|---|---|---|
| $\log K_{ow}$ | 2.130 | 2.690 | 3.150 | 2.770–3.120 | 3.200 | 3.150 |
| $w_{vaq}$ | 0.178 | 0.0627 | 0.0152 | 0.0175 | 0.0196 | 0.019 |

Aerobic degradation of these compounds is usually initiated by progressive oxidation of the alkyl side chain of the aromatic ring to produce carboxylic acids, or ring oxidation which produces substituted pyrocatechols. Carboxylic acids and pyrocatechols are then transformed to substrates of the citrate cycle through cleavage of the aromatic ring.

Anaerobic metabolism is important, because BTEX are frequently found under oxygen-limiting conditions in sediments, groundwater and soil. No single organism has been reported to mineralize benzene completely under anaerobic conditions. However, it has been shown to be degraded anaerobically by enriched mixed culture. Both toluene and ethylbenzene have been shown to have a common metabolic intermediate, benzoyl-CoA, which is the most frequent central intermediate of anaerobic aromatic metabolism (Heider *et al.* 1997). The aromatic ring of benzoyl-CoA is reduced and eventually transformed to acetyl-CoA. Few organisms are capable to metabolize xylene anaerobically. They include strains of denitrifying bacteria capable of using *m*-xylene as growth substrate (Harwood *et al.* 1997). This review will focus on aerobic metabolism of BTEX.

## 2 AEROBIC DEGRADATION OF BTEX

Two bacterial multicomponent enzymic systems, monooxygenases and dioxygenases, were found to be responsible for the degradation of BTEX in the environment. Monooxygenases produce arene oxides as intermediates and use only one oxygen atom from the oxygen molecule during oxidation. They must be coupled with a hydratase or they need the presence of peroxide to produce *trans*-diols. Toluene–xylene monooxygenases of *Pseudomonas mendocina* KR1 and *Burkholderia cepacia* G4 use non-heme iron as electron acceptor. Dioxygenases were found only in bacteria and generate peroxide intermediates, which are spontaneously transformed into *cis*-diols. They use both oxygen atoms from an oxygen molecule. Dioxygenases are non-heme $Fe^{3+}$-containing enzymes and more substrate-specific than monooxygenases. Stapleton *et al.* (1998) have used several DNA probes to understand the distribution of known enzymic systems in the jet-fuel field. The average percentage of community values were 10.8 % *alkB* (alkane hydroxylase), 7.6 % *nahA* (naphthalene dioxygenase), 11.1 % *nahH*/*xylE* (pyrocatechol-2,3-dioxygenase), 7.3 % *todC1C2* (toluene dioxygenase), 5.3 % *tomA* (toluene monooxygenase) and 2.5 % *xylA* (xylene monooxygenase) genotypes.

### 2.1 *Transformation by monooxygenase*

The genes encoding the best-known toluene–xylene degradation pathway (Worsey *et al.* 1975) employing a monooxygenase are carried on TOL plasmids. The plasmids were isolated by different laboratories around the world. Although these TOL plasmids vary in size, incompatibility group, genetic organization of catabolic genes and other characteristics, they encode pathways that are very similar. The archetypal TOL plasmid was designated pWWO (Harayama *et al.* 1989). On the TOL plasmid pWWO, one operon (*xylUWCMABN*) codes for the "upper" pathway enzymes, which oxidize methylbenzenes to methylbenzoates (*xylU*, *xylW* and *xylN* gene products are not required for growth on toluene and xylene) while the "lower" pathway or *meta*-operon is composed of 13 genes (*xylXYZLTEGFJQKIH*) for enzymes converting methylbenzoates to pyruvate, acetaldehyde and acetate *via* (methyl)pyrocatechols. In addition to the catabolic operons, two regulatory genes, *xylS* and *xylR*, located on the 3′ end from *meta*-operon, are involved in transcriptional control. The mechanism of gene induction is described by Ramos *et al.* (1997). *P. putida* mt-2 strain is using a pathway carried on the TOL plasmid. Besides utilizing toluene (Fig. 1; **1** where R is $CH_3$), it grows also on *m*-xylene (**1**, where R is 1-$CH_3$, 3-$CH_3$), *p*-xylene (**1**, where R is 1-$CH_3$, 4-$CH_3$), *m*-ethyltoluene (**1**, where R is 1-$CH_3$, 3-$CH_3CH_2$), and 1,2,4-trimethylbenzene (**1**, where R is 1-$CH_3$, 2-$CH_3$,

4-$CH_3$). These substrates are all oxidized to corresponding benzylalcohols (**12**), benzaldehydes (**13**) and benzoates (**14**) and the *meta*-cleaved to corresponding substituted pyrocatechols (**8**), and subsequently mineralized by *meta*-cleavage pathway (Fig. 2) (Williams *et al.* 1974, Murray *et al.* 1972).

*Nitrosomonas europea* ATCC19718 is an obligate autotroph. It uses oxygen as electron acceptor and ammonia as its sole source of energy. BTEX are oxidized by *Nitrosomonas* aminomonooxygenase (AMO), which is activated by ammonia. Benzene (**18**) is oxidized to phenol (**19**) and then to hydroquinone (**20**). This reaction inhibits oxidation of ammonia (Hyman *et al.* 1985). Ethylbenzene (**21**) is oxidized by AMO to several products: 1-phenyl-1-ethanol (64 %) (**25**) which is consequently degraded to acetophenone (**26**) and hydroxyacetophenone (**27**). The latter is degraded to phenylglyoxal (**28**). 4-Ethylphenol (4 %) (**24**) and styrene (**22**) are other intermediates. Toluene (**1**, where R is $CH_3$) is oxidized to benzylalcohol (**12**), then to benzaldehyde (**13**). *p*-Xylene (**1**, where R is 1-$CH_3$ and 4-$CH_3$) is oxidized to 4-methylbenzylalcohol (**12**, where R is 4-$CH_3$) then to *p*-tolualdehyde (**13**, where R is 4-$CH_3$) and/or to 1,4-benzenedimethanol (**17**) (Keener *et al.* 1994).

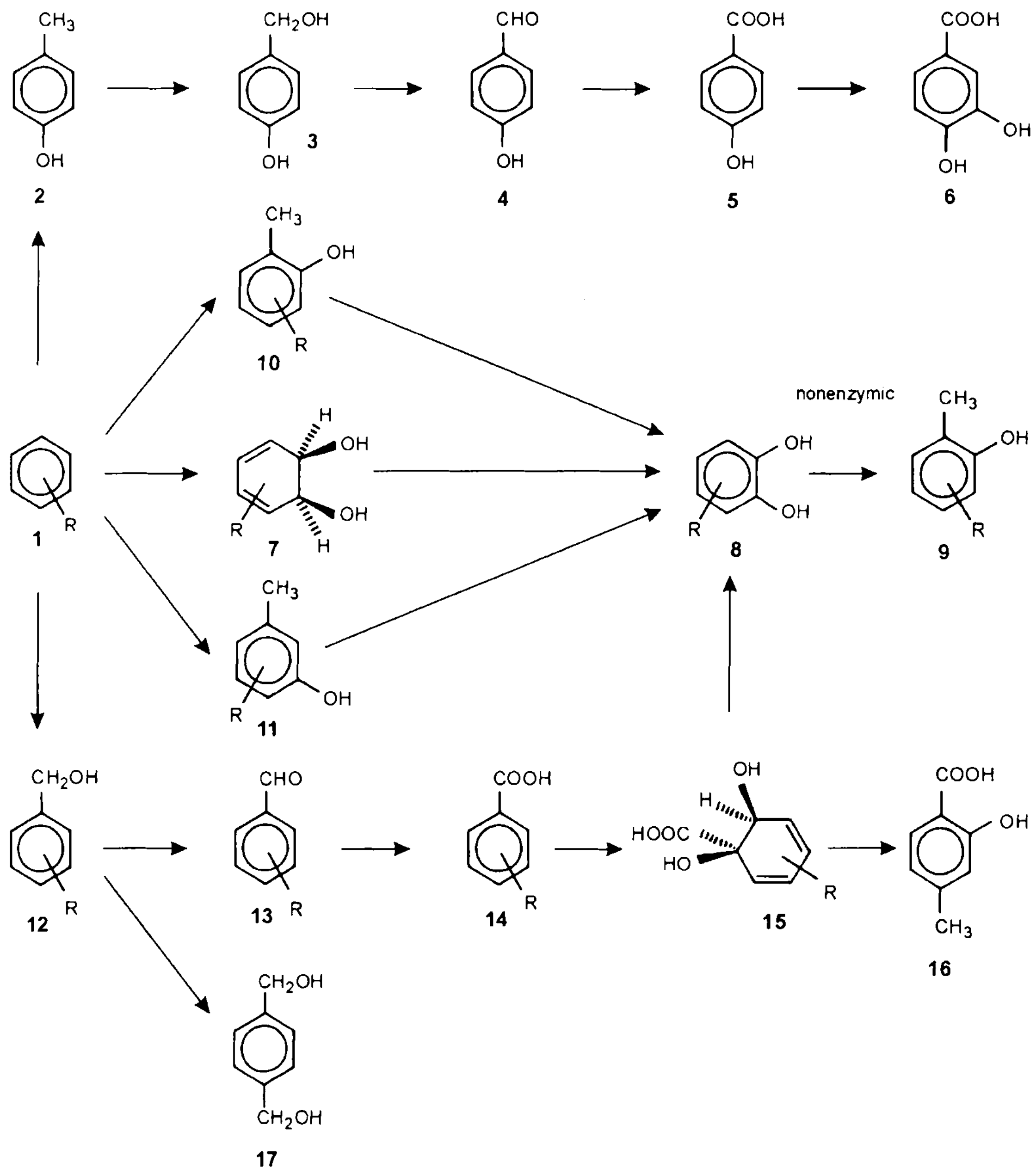
*Pseudomonas* sp. P-X and M-X, respectively, were isolated from a muddy pond in Pennsylvania by cultivation on *p*-xylene (P-X) and *m*-xylene (M-X) as the sole source of carbon and energy. The methyl group of *p*-xylene (**1**, where R is 1-$CH_3$ and 4-$CH_3$) is oxidized by P-X strain to *p*-toluate (**14**, where R is 4-$CH_3$), which is converted by oxidization and decarboxylation to 3- or 4-methylpyrocatechol. *m*-Xylene (**1**, where R is 1-$CH_3$ and 3-$CH_3$) is oxidized to *m*-toluate (**14**, where R is 3-$CH_3$) and 3-methylpyrocatechol (**8**, where R is 3-$CH_3$). Methyl pyrocatechol is then mineralized by *meta*-cleavage (Fig. 2) to hydroxymuconaldehydate which can be converted to 2-pyridinecarboxylate in the presence of ammonia (Davis *et al.* 1968).

*Pseudomonas aeruginosa* S668B2 produces 4-methylpyrocatechol (**8**, where R is 4-$CH_3$) when growing on *p*-xylene (**1**, where R is 1-$CH_3$, 4-$CH_3$). The main product of *m*-xylene (**1**, where R is 1-$CH_3$, 3-$CH_3$) metabolism is 4-methylsalicylic acid (**16**), which is not further oxidized by this strain (Davey *et al.* 1974).

*Pseudomonas* sp. Pxy was isolated from soil taken from the edge of a polluted creek at Austin (Texas) with *p*-xylene as the sole carbon source. It can grow on *p*-xylene, *m*-xylene, 4-methylbenzylalcohol, and it oxidizes potential intermediates of their metabolism, *e.g.*, *p*- and *m*-tolualdehyde substrates; *p*-, *m*-toluate; 3- and 4-methylpyrocatechol (**8**). However, *Pseudomonas* sp. strain Pxy is not able to oxidize *o*-xylene, benzene and cresol isomers. The substrates *p*- and *m*-xylene (**1**, where R is 1-$CH_3$, 4-$CH_3$ and 3-$CH_3$, respectively) are oxidized by $NAD^+$ to *m*- and *p*-toluic acid (**9**, where R is 3-$CH_3$ and 4-$CH_3$, respectively) and to 3- and 4-methylpyrocatechol (**8**, where R is 3-$CH_3$ and 4-$CH_3$, respectively). Mutant strains *Pseudomonas* sp. Pxy-40 and Pxy-82, which lost the ability to grow on *p*- and *m*-xylene, respectively, were also studied. In a succinate culture of Pxy-40, *p*-toluate was found as a metabolite in the presence of *p*-xylene in media. No metabolites were detected in the presence of *m*-xylene in the media. It suggests that different enzyme systems are responsible for further oxidation of *p*- and *m*-toluic acid. 4-Methylpyrocatechol (**8**) was isolated from the culture of Pxy-82 in the presence of *p*-xylene and it is rapidly oxidized by the wild type of the strain (Davey *et al.* 1974).

Aromatic compounds that can serve as growth substrates for *P. putida* Idaho include toluene, *m*-xylene, *p*-xylene, 1,2,4-trimethylbenzene (pseudocumene), 3-ethyltoluene, benzylalcohol, benzoic acid, *m*-toluic acid, *p*-toluic acid, 4-hydroxybenzylalcohol, *m*-cresol and *p*-cresol (Cruden *et al.* 1992). This strain has the same growth phase in the presence of *p*-xylene (5–50 %, *V/V*) and uses the same metabolic pathway as *P. putida* mt-2 for the degradation of alkyl-substituted aromatic hydrocarbons. It oxidizes any of these aromatic hydrocarbons to corresponding benzylalcohols (**12**) and carboxylic acids (**14**), which are formed by oxidation of the methyl group. Genes encoding enzymes for the degradation of toluene, *m*-xylene and *p*-xylene are located on the chromosome of *P. putida* Idaho. High similarity with *xylCMA* genes of *P. putida* mt-2 was found by DNA–DNA hybridization of total DNA from *P. putida* Idaho and a DNA probe of *P. putida* mt-2 (Cruden *et al.* 1992).

*Pseudomonas paucimobilis* Q1 can grow on biphenyl, *m*-, *p*-xylene, toluene or both xylene and toluene, salicylate and octane. It also grows on *m*-methylbenzylalcohol (**12**) and *m*-tolualdehyde (**13**). Toluene is oxidized (**1**, where R is $CH_3$) to benzylalcohol (**12**), further to benzaldehyde (**13**) and benzoate (**14**); and *m*-, *p*-xylene (**1**, where R is 1-$CH_3$, 3- and 4-$CH_3$, respectively) to *m*-, *p*-toluate (**14**), then to corresponding pyrocatechols (**8**), which are mineralized by *m*-cleavage (Fig. 2). Benzene- and toluene-oxidizing activity of strain Q1 was induced by *m*-xylene and its metabolites, such as 3-methylbenzylalcohol, *m*-tolualdehyde and *m*-toluate, biphenyl and salicylate. *m*-Xylene-oxidizing activity was induced when strain Q1 grew on biphenyl, salicylate and *m*-toluate. Upon hybridization of total DNA, strain Q1 has shown no significant homology with SAL and TOL plasmids (Furukawa *et al.* 1983).




*P. mendocina* KR1 uses a multicomponent enzyme system which catalyzes a monooxygenase reaction. The ability of the enzyme is dependent on the source of reducing equivalents (NADH or NADPH) and oxygen. Toluene monooxygenase is a multicomponent system (Whited *et al.* 1991), which transforms toluene to *p*-cresol (**2**).

*p*-Cresol is then oxidized by several oxidations of the methyl group to protocatechuic acid (**6**), which is further mineralized through *ortho*-cleavage (Fig. 2). Toluene monooxygenase genes were designed *tmoA*, *tmoB*, *tmoC*, *tmoD*, and *tmoE*. Immediately upstream from the TMO gene cluster there are two putative open reading frames running in the same direction as *tmo* genes. The GC content of *tmo* genes indicates that these genes originated from another bacterial species and were later transferred to *P. mendocina* on a plasmid (Yen *et al.* 1991).

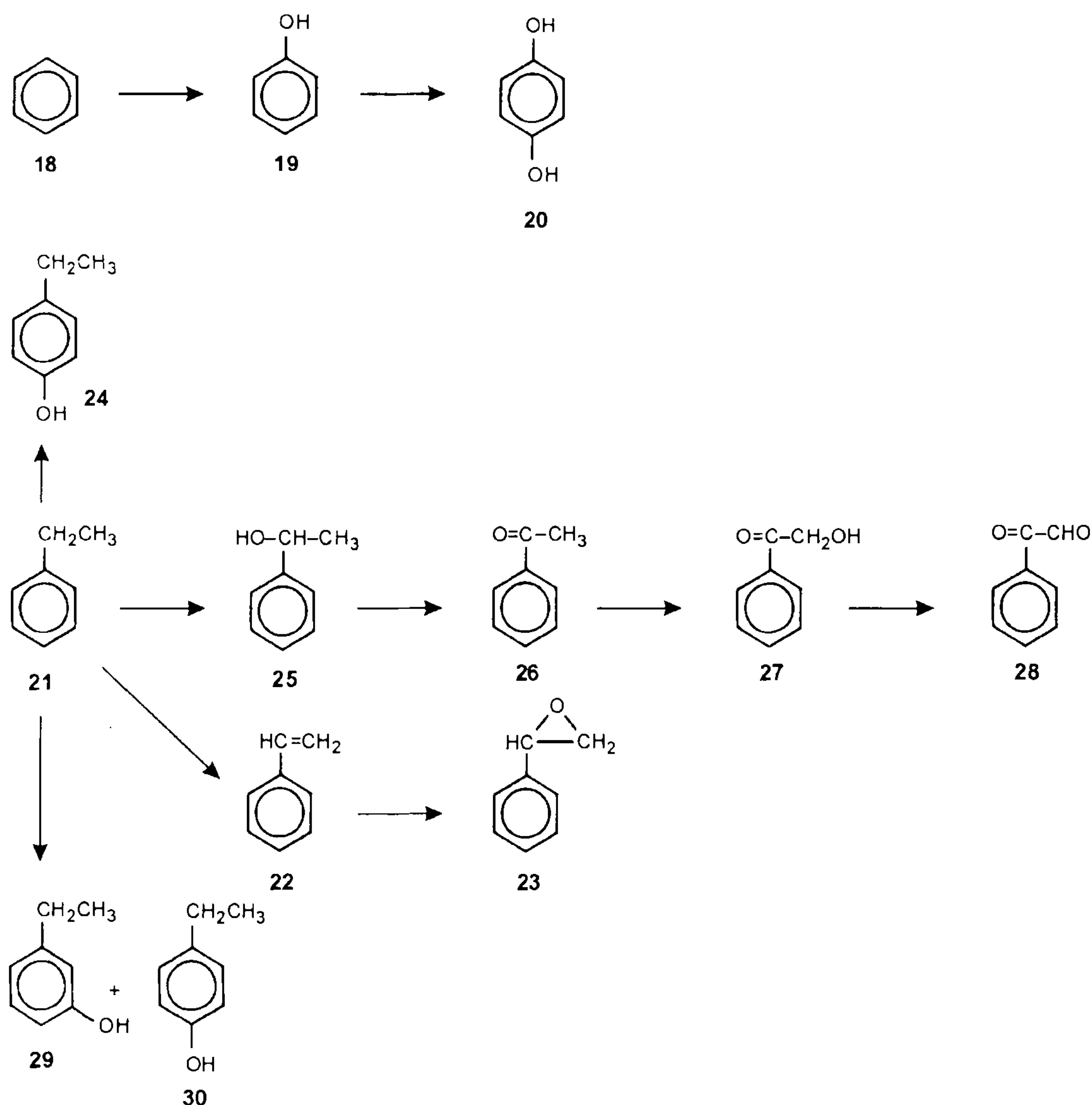



Fig. 1. Transformation of BTEX through alternative metabolic pathways ("upper" metabolic pathways); R – alkyl substituents linked at 3, 4 and/or 5 position, respectively.

Toluene-3-monooxygenase from *Pseudomonas pickettii* PKO1 not only catalyzes the conversion of toluene to *m*-cresol (**11**) but also converts benzene (**18**) to phenol (**19**), and ethylbenzene (**21**) to 3-ethylphenol (**29**) and 4-ethylphenol (**30**). Moreover, after induction by toluene, *o*-, *m*- and *p*-xylene are converted to the corresponding methylphenols. However, *p*-xylene and ethylbenzene were poorly transformed. *m*-Xylene is oxidized to 2,4-dimethylphenol (**10**, where R is 4-$CH_3$) and not to 3,5-dimethylphenol (**11**, where R is 5-$CH_3$), as might be expected from toluene-3-monooxygenase. Gene organization of the PKO1 pathway seems to be different from the organization of other known toluene pathways and their monooxygenases (*e.g.*, the pathways located on TOL plasmids and loci encoding toluene monooxygenase of *P. mendocina* KR1). Toluene monooxygenase of *P. picketii* PKO1 is a multicomponent system such as the TOL plasmid and *P. mendocina* KR1 toluene monooxygenase (Olsen *et al.* 1994).

### 2.2 *Transformation by dioxygenase*

Genes for benzene and toluene utilization (*tbu*) of the strain *Pseudomonas pickettii* PKO1 are located on the chromosome. They are designated *tbuABC*, *tbuJHIKGFE*, *tbuD* as structural genes and *tbuR* and

*tbuS* as regulatory genes of toluate-3-dioxygenase. *tbuC* represses transcription of *tbuEFGKIHJ* in the absence of effectors. The effectors, phenol and *m*-cresol, interact with *tbuR* and *tbuS* to form complexes that activate transcription of *tbuD* (oxidation of *m*-cresol) and *tbuEFGKIHJ* (*m*-cleavage of pyrocatechol) (Olsen *et al.* 1994).

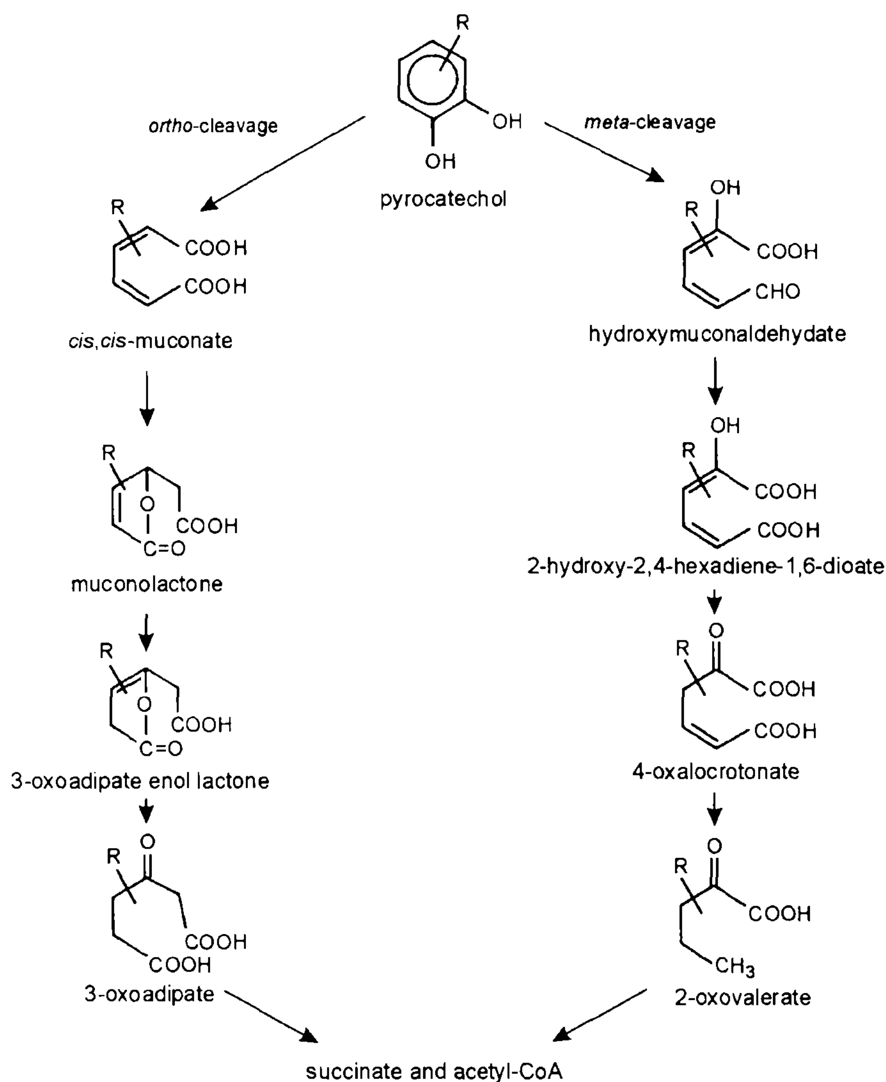



**Fig. 2.** Metabolism of pyrocatechol ("lower" metabolic pathways); R – alkyl substituents linked at 3, 4 and/or 5 position, respectively.

Strain *P. putida* F1 first metabolizes toluene (**1**, where R is $CH_3$) to *cis*-1,2-dihydrotoluene-1,2-diol (**7**, where R is 3-$CH_3$) by toluene dioxygenase and this it is followed by conversion to 3-methylpyrocatechol (**8**, where R is 3-$CH_3$) which undergoes *meta*-cleavage. The nucleotide sequence of the *todC1C2BADE* cluster encoding enzymes of the toluene degradation pathway (*todC1C2-ISP*, *todB* – ferredoxin, *todA* – reductase, *todD* – dihydrodiol dehydrogenase, *todE* – 2-methylpyrocatechol-2,3-dioxygenase) was characterized. The *todC1C2BAD* genes show significant homology with genes encoding benzene dioxygenase and *cis*-1,2-dihydroxycyclohexa-3,5-diene dehydrogenase from *P. putida* 136R-3. In addition, significant homology between *todDE* and sequences reported for *cis*-1,2-dihydroxy-6-phenylcyclohexa-3,5-diene dehydrogenase and 2,3-dihydroxybiphenyl-1,2-dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 was observed (Zylstra *et al.* 1989).

*P. putida* 39/D was selected by prolonged cultivation of the wild-type strain which is able to utilize benzene, and oxidize benzene, toluene, ethylbenzene, 1-phenyl-1-ethanol, acetophenone, 4-halotoluene, chlorobenzene, and benzonitrile to *cis*-dihydrodiol derivatives (Kobal *et al.* 1973, Ziffer *et al.* 1973). It does not grow on xylene isomers. *P. putida* 39/D can convert *p*-xylene (**1**, R is 1-$CH_3$, 4-$CH_3$) to *cis*-3,6-dimethyl-

3,6-cyclohexadiene-1,2-diol (**7**, where R is 3-$CH_3$ and 6-$CH_3$) which is accumulated by growing on succinate. *m*-Xylene is converted to 3,5-dimethyl-3,5-cyclohexadiene-1,2-diol (**7**, where R is 3-$CH_3$ and 5-$CH_3$), which is rapidly converted to 2,4-dimethylphenol (**10**, where R is 4-$CH_3$). Wild type of *P. putida* is able to produce 3,6-dimethylpyrocatechol (**8**, where R is 3-$CH_3$, 6-$CH_3$) and 3,5-dimethylpyrocatechol (**8**, where R is 3-$CH_3$, 5-$CH_3$) by growing on succinate. The latter products were not isolated. The ability to grow on dimethylated substituents indicates a degree of dioxygenase ability to catalyze *meta*-cleavage (Fig. 2). 3,6-Dimethylpyrocatechol was further detected as an intermediate of *p*-xylene oxidation by strain *Nocardia corallina* V-49, which is transformed to 2,4-dimethyl-*cis,cis*-muconic acid by *ortho*-cleavage (Gibson *et al.* 1974).

*Pseudomonas putida* ML2 utilizes benzene as the sole carbon and energy source. Benzene dioxygenase transforms benzene to *cis,cis*-3,5-cyclohexadiene-1,2-diol (7). Using the heterologous toluene dioxygenase (*tod*) genes from *P. putida* F1 as probes, the benzene dioxygenase genes were found to be encoded on a 112 kb plasmid, pHMT112 (*Pseudomonas putida* ML2). They were identified as benzene dioxygenase genes (*bedABC1C2*) by cloning and subsequent gene expression in *E. coli* (Tan *et al.* 1990). Four polypeptides of benzene dioxygenase are homologous with toluene dioxygenase but not with naphthalene dioxygenase.

Benzene utilization was studied in both Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Mycobacterium rhodochrous*. Benzene is converted by these organisms to 3,5-cyclohexadiene-1,2-diol (7), then to pyrocatechol (**8**), which is degraded through *cis,cis*-muconic acid and 3-oxoadipate to succinate and acetyl-CoA (Fig. 2) (Marr *et al.* 1961).

### *2.3 BTEX degraders isolated by enrichment on other substrates*

Lang *et al.* (1996) have studied diversity of bacteria capable to utilize aromatic compounds. Seventeen strains were obtained in the *German Collection of Microorganisms and Cell Cultures GmbH*, Braunschweig, and tested for their ability to degrade 15 additional aromatic substrates. A relatively high number of additional positive results was found for the substrates benzene, toluene, and for the versatile strain *Sphingomonas* sp. DSM 6900. On the other hand, no additional strains metabolizing *o*-xylene were found. *o*-Xylene was utilized only by strains that were isolated by using a specific compound as a carbon source. Seventy and 42 % of the isolates metabolized toluene and *p*-xylene, respectively, as their sole source of carbon, while only 15 % were able to grow on *o*-xylene. Most of the strains degrading *m*-xylene are also able to metabolize another compound. The utilization patterns of the aromatic compounds were highly diverse. In this study it was found that benzenes with more than one alkyl substituent tend to be degraded by Gram-negative bacteria (Lang *et al.* 1996).

The ability of *P. putida* BP18, which was isolated on the basis of its ability to utilize either biphenyl or naphthalene as the sole carbon source, to utilize BTEX was tested by Baldwin *et al.* (2000). The strain utilizes benzene, toluene and ethylbenzene as the sole carbon source but utilization of xylene was not observed. A biphenyl-grown strain transformed all substrates, *i.e.*, benzene, toluene, ethylbenzene, *o*-, *m*- and *p*-xylene and the concentration of BTEX compounds was below the detection limit. It suggests a broad specificity for the aromatic metabolic pathway (cf. Marek *et al.* 2001; Weigner *et al.* 2001).

Purified naphthalene dioxygenase (NDO) from *Pseudomonas* sp. NCIB 9816-4 oxidized toluene to benzylalcohol and benzaldehyde by a reaction involving benzylic monooxygenation and dioxygen-dependent alcohol oxidation. Ethylbenzene is degraded to 1-phenyl-1-ethanol, then to acetophenone and finally to 2-hydroxyacetophenone, with secondary pathway involving the formation of styrene (**22**) and phenyl-1,2-ethanediol. NDO catalyzes the oxidation of xylenes and nitrotoluenes to corresponding alcohols and aldehydes, whereas toluene dioxygenase (TDO) from *P. putida* F1 oxidizes *m*- and *p*-xylene to *cis*-dihydrodiols and does not oxidize *o*-xylene (Lee *et al.* 1996).

## 3 EFFECT OF PHYSICAL AND CHEMICAL FACTORS ON BACTERIAL DEGRADATION OF BTEX

*Oxygen.* Toluene degrading strains were tested under hypoxic conditions. For example the degradation of toluene by *P. pickettii* PKO1 was enhanced in the presence of nitrate. When TOL plasmid pWWO was transferred by conjugation from the strain PaW1, which is not active under hypoxic conditions, into two archetypal denitrifying strains *P. denitrificans* ATCC 19367 and *P. stutzeri* ATCC 17588, the conjugates were able to grow on toluene as the sole carbon source when inoculated under aerobic conditions. No degradation was observed under hypoxic conditions (Kukor *et al.* 1996).

*Surfactants.* The influence of the non-ionic surfactant Triton X-100 and another pollutant on BTEX was tested. Application of Triton X-100 up to 400 mg/L was not expected to enhance significantly the solubility of relatively soluble BTEX compounds (Lee *et al.* 2000).

*Solvents.* The effect of *p*-xylene and toluene on succinate-grown cells of *P. putida* strain Idaho was studied. When cells are grown with *p*-xylene vapors, the outer membrane is convoluted and on places clearly separated from the cytoplasmic membrane. Inclusion bodies were different from those seen in succinate-grown cells. When cells are grown with 20 % *p*-xylene, the outer and cytoplasmic membranes are separated and convoluted and especially the cytoplasmic membrane appears disorganized. However, higher distribution of membranes is apparent with vapors and with 20 % toluene than with *p*-xylene (Cruden *et al.* 1992; Pinkart *et al.* 1996).

*Water potential.* The influence of water potential on growth rates of *P. putida* mt-2 was tested. The presence of NaCl and PEG-8000 had a stronger effect in a toluene-grown culture than in a succinate-grown one. A decrease of water potential by NaCl did not significantly influence the toluene utilization rate coefficient (Holden *et al.* 1997).

*Substrate interactions.* Few studies have been conducted in an effort to understand substrate interactions of monoaromatics in a mixture. Mixed consortium and single species were used for these studies. For example Oh *et al.* (1994) used a mixed consortium and strain *Pseudomonas* sp. PPO1 and found that benzene and toluene can be degraded individually, *p*-xylene cannot, while in a mixture of benzene, toluene, and *p*-xylene, respectively; benzene and toluene are degraded according to competitive inhibition kinetics, whereas *p*-xylene was partially removed by co-metabolism. Burback and Perry (1993) reported that *Mycobacterium vaccae* could individually catabolize benzene or toluene, and also a mixture of the compounds. While benzene degradation is enhanced, toluene degradation rate is not changed. Alvarey and Vogel (1991) suggest that the presence of toluene in mixtures with benzene and *p*-xylene enhanced the degradation of both aromatics. This research observed benzene-dependent degradation of toluene and *p*-xylene, and retardation of both benzene and toluene degradation by *p*-xylene, which is co-metabolically degraded. Strains *Pseudomonas* B1 and X1, resp., were used for the quantification of competitive inhibition kinetics of a mixture of benzene–toluene, benzene–*p*-xylene, and toluene–*p*-xylene (Chang *et al.* 1992). Competitive inhibition in a toluene- benzene mixture and co-metabolic degradation of *p*-xylene by growth on toluene was observed. Arvin *et al.* suggested, by studying two consortia, that the presence of either toluene or *o*-xylene stimulates benzene degradation. However, when benzene was present in a mixture with both toluene and *o*-xylene, the stimulation effect was smaller.

Toluene-enrichment consortium and *Rhodococcus rhodochrous* were used for the interaction study of BTEX. Toluene-enrichment culture degrades toluene faster than benzene, followed by ethylbenzene and xylene. The presence of *o*-xylene enhanced the degradation of benzene or toluene. The presence of either toluene, benzene or ethylbenzene had a negative effect on xylene degradation rates. When the degradation of BTEX mixture was studied, ethylbenzene was degraded fastest, followed by benzene, toluene and xylene. The degradation rate of toluene and benzene in BTEX mixtures was retarded by the presence of benzene or toluene, respectively. Ethylbenzene was the most potent inhibitor of benzene, toluene and xylene degradation by *R. rhodochrous* culture in all tested substrate mixtures (Deeb *et al.* 1999).

The next example of substrate interaction is described by Bielefeldt *et al.* (1999) who used a mixed culture with the ability to degrade a wide range of aromatics (phenol, BTEX and naphthalene). A degradation test showed that the degradation rate of phenol was the fastest followed by toluene, benzene, ethylbenzene, *p*-xylene, *o*-xylene and naphthalene. A biodegradation test of the BTEX mixture revealed competitive inhibition for multiple compounds causing a lower degradation rate. Despite the inhibition effect, the ability of the mixed culture to degrade a mixture of compounds may be important for a variety of waste treatment applications.

*Soil influence.* Zhang *et al.* (1997) studied the influence of the presence of soil in aqueous solution on the growth and degradation rate of benzene, toluene and naphthalene. They found three aspects. In the presence of soil, biodegradation lasted longer due to long *lag* phases. Significant biodegradation in soil-water microcosms was not detected until after 3 d while biodegradation in water started between within a day. The rates of biodegradation were significantly lower in the presence of soil. Relative rate (sequence) of biodegradation shifted from toluene > naphthalene > benzene in the water and to toluene > benzene > naphthalene in soil-water slurry mixtures.

*Immobilization of cells.* The growth rate and degradation rate increased with increasing concentration of the substrate until reaching a maximum value. Then it decreased with increasing concentration of the substrate which indicated substrate inhibition. Kinetic studies of BTEX biodegradation by the co-culture of *P. putida* and *P. fluorescens* described by Shim *et al.* (1999) showed the lowest limiting concentration of benzene to be 500 mg/L. Immobilized cells in fibrion-bed bioreactor showed that BTEX can be effectively

degraded under hypoxic conditions and with high substrate concentrations. Immobilized cells adapted in the bioreactor were less sensitive and more tolerant to toxic substrates than mobile ones (Shim *et al.* 1999).

## 4 NATURAL ATTENUATION OF BTEX CONTAMINATED SITES

Aerobic mineralization of benzene requires approximately 3 mg $O_2$ per mg degraded benzene. Under ambient conditions, water contains only 8–12 mg/L dissolved oxygen, which is sufficient for degradation of only 3–4 mg/L of BTEX unless aeration or other sources of oxygen are also provided or biodegradation takes place *via* anaerobic processes. Twenty-five mL of air in headspace would provide an equivalent amount of 75 mg/L $O_2$ in the liquid medium (100 mL) in a serum bottle. These experiments clearly showed that the co-culture was not able to efficiently degrade highly concentrated BTEX under hypoxic conditions in a serum bottle. Addition of $H_2O_2$ as additional oxygen source in water has been suggested to overcome the oxygen limitation problem. However, the degradation test cannot be characterized only by $CO_2$ and $O_2$ uptake but also by distinguishing microbial degradation activity from the other processes; it is necessary to determine parameters like concentrations of the contaminants, electron acceptors and metabolites in degradation assays as is described by Althoff *et al.* (2001) in the potential for natural attenuation of contaminated groundwater. Counts of aerobic bacteria in the original groundwater and in the assays at the end of the test do not relate to the observed microbial activity. Therefore, natural attenuation at this former gas plant site with just a small indigenous microflora would not be successful as a remediation strategy without ammonium and phosphate supplementation.

Kao *et al.* (2001) observed a correlation between natural attenuation and natural biodegradation. Natural attenuation rates for BTEX were only slightly higher than natural biodegradation rates. This indicates that natural biodegradation processes are a major cause for BTEX removal. Without occurrence of natural biodegradation, other natural attenuation processes cannot reduce contamination effectively. Under a methanogenic biodegradation process, toluene had the highest decay rate among the BTEX, and *o*-xylene had the lowest rate. The degradation trend for BTEX decreased as follows toluene, *p*-xylene, benzene, ethylbenzene, and *o*-xylene. Results from other studies have shown that the BTEX degradation trend varied from site to site. Environmental factors and the status of biodegradation processes such as methanogenesis, iron reduction, denitrification, sulfate reduction and aerobic biodegradation would cause the variation in BTEX degradation order. Up to 89 % of BTEX was removed in natural processes. It suggests that natural attenuation could be used as a remedial option at the gasoline spill site to save the contaminated groundwater. Higher BTEX concentrations within this area might inhibit the microbial biotransformation processes, which cause the decrease of degradation efficiency. However, other environmental factors (*e.g.*, local subsurface heterogeneity, microbial populations, variation in dissolved oxygen concentration, status of oxidation–reduction, might also affect the natural attenuation and biodegradation rates (Kao *et al.* 2001).

An example of natural attenuation is described by Cho *et al.* (1997) and Häner *et al.* (1995), who documented the contribution of sulfate reduction, $Fe^{3+}$ reduction, aerobic respiration, and denitrification, respectively, to remove BTEX compounds from groundwater by geochemical analysis of groundwater after 8 months of active remediation at JP-4 jet-fuel spilled field. The total dissolved electron acceptors available in groundwater exceeded the concentration of BTEX compounds in groundwater at all times. It indicates that the rate of natural biodegradation was controlled by the rate of the electron acceptor supply present in non-contaminated groundwater moving toward the residual light non-aqueous phase liquids (Kao *et al.* 2001).

In conclusion, BTEX degradation may potentially be suggested for feasible natural attenuation processes but the disputed complex set of data should always be gathered and analyzed to achieve site-specific remediation objectives within the time frame that is reasonable compared to that offered by more actual methods.

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