Bacterial degradation of phthalate isomers and their esters

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Received: 31 July 2007 / Final revision: 3 October 2007 / Accepted: 19 October 2007

Abstract Phthalate isomers and their esters are used heavily in various industries. Excess use and leaching from the product pose them as major pollutants. These chemicals are toxic, teratogenic, mutagenic and carcinogenic in nature. Various aspects like toxicity, diversity in the aerobic bacterial degradation, enzymes and genetic organization of the metabolic pathways from various bacterial strains are reviewed here. Degradation of these esters proceeds by the action of esterases to form phthalate isomers, which are converted to dihydroxylated intermediates by specific and inducible phthalate isomer dioxygenases. Metabolic pathways of phthalate isomers converge at 3,4-dihydroxybenzoic acid, which undergoes either *ortho*- or *meta*- ring cleavage and subsequently metabolized to the central carbon pathway intermediates. The genes involved in the degradation are arranged in operons present either on plasmid or chromosome or both, and induced by specific phthalate isomer. Understanding metabolic pathways, diversity and their genetic regulation may help in constructing bacterial strains through genetic engineering approach for effective bioremediation and environmental clean up.

Keywords Phthalate esters · Toxicity · Biodegradation · Dioxygenases · Genetic regulation

Introduction

Since last seven to eight decades phthalate, terephthalate, isophthalate and their esters are used as plasticizers [57] (Fig. 1). They have low water solubility but high octanol/water partition coefficients (K_{ow}) and the hydrophobicity increases with increase in alkyl chain length [2, 10, 57]. Because of their unique properties like chemical stability, flexibility, softening, ease of fabrication and low cost, phthalate esters are used with different chemical formulations. Phthalates and their esters are the major constituents of paints, house building materials, PVC pipes, food packing, toys, plastics, adhesives, photography films, textile fabrics, medical devices, pesticide carriers, defoaming agents in the manufacture of paper, lubricating oils, solubilizers of cosmetic products, and in aerospace technology [34, 38, 41, 44, 55, 65, 97, 98, 104, 106].

With an annual production of 14.4 million ton, dimethyl terephthalate and terephthalate are among the top 50 most produced chemicals worldwide [58]. These plasticizing agents are not linked by covalent interactions to the parent chemical, imparting flexibility to the product and are leached out easily by organic and polar solvents [2, 38, 40, 45, 97]. Besides synthetic origin, few of these compounds are metabolic intermediates in the degradation of polyaromatic hydrocarbons, for example, in several bacterial species *o*-phthalate was shown to be an intermediate in phenanthrene, pyrene, fluorine and fluoranthrene catabolism [37, 42, 48, 56, 68, 79]. Bioaccumulation of these compounds in nature led to adaptation and evolution of new metabolic

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Fig. 1 Few representative chemical structures of different phthalate isomers and their esters used in the industry.

pathways for the degradation of these compounds. Therefore characterization and understanding the metabolic processes in these microbes can be exploited for the efficient and economic removal of these compounds from nature through bioremediation.

Phthalate toxicity

The major source of human exposure to phthalate isomers and their esters is by medical, occupational and dietary products [57]. Humans exposed to phthalate isomers showed teratogenic, estrogenic and reproductive problems [22, 24, 34, 44, 54, 86]. Small chain alkyl esters of phthalates are shown to be more toxic because of their high solubility compared to longer chain homologs. Exposure during the development results in irreversible damage [17]. These compounds suppress calcium signaling and elicit cellular oestrogenic responses [69, 61, 69, 60]. In mice these

compounds induced malformations of the reproductive tract [36, 59] causing hypospadias and cryptorchidism [31, 77]. They are detected in body fluids [46, 110] and are known to induce bladder stone formation, irritation of skin, eyes, respiratory tract and blurred vision [20, 21, 103]. In humans, phthalate esters are biotransformed, conjugated to glucuronides and either excreted or stored in body [46, 57].

Among all the phthalate isomers, isophthalate is reported to be a competitive inhibitor of glutamate dehydrogenase (GDH) of yeast, *Aspergillus*, bovine and pea plant [7, 19, 85]. GDH is an anapleurotic enzyme that catalyses the conversion of α -ketoglutarate to glutamate and *vice versa* [43]. The inter-proton distance between the two carboxylate groups of isophthalic acid is same as that of α -ketoglutarate and iminoglutarate (reaction intermediate), thus making it an ideal competitive inhibitor of the enzyme [7]. Phthalate esters also inhibit 15-lipoxygenase [63] involved in the fatty acid metabolism.

Bacterial metabolism of phthalate isomers and their esters

Phthalate isomers and their esters are present in the environment. Due to persistence in the nature, microbes have evolved and adapted new pathways to degrade these compounds. Bacterial degradation is efficient as these compounds are either biotransformed or mineralized completely. The degradation of phthalate isomers can be both by aerobic and anaerobic routes. Many bacterial strains have been reported to degrade phthalate esters as the sole carbon source (Table 1), while some strains like *Rhodococcus rhodochrous* and *Closterium lunula* need external addition of hexadecane or inorganic carbon for efficient degradation [70, 107].

Anaerobic degradation

The anaerobic phthalate degradation is mainly carried out by methanogenic consortia (Table 1). Bacteria degrade all three phthalate isomers and their esters producing acetate and methane as the end products [8, 28, 52, 53, 82]. The anaerobic route for degradation is shown in Fig. 2. The first and the foremost step in the degradation of phthalate isomers is the production of esters of CoA by an inducible acyl CoA synthetase [74]. Decarboxylation of these esterified phthalates by decarboxylase results in the formation of benzoyl CoA [51, 74, 82]. In few cases phthalates are first decarboxylated in the presence of specific decarboxylases followed by esterification with CoA [53]. Reduction of benzoyl CoA produces cyclohex-1-ene-carboxylate ester of CoA. This product undergoes ring cleavage and form pimelyl CoA via 2-oxocyclohexanecarboxylate and enters β -oxidation pathway [74] (Fig. 2). External addition of acetate, pyruvate or lactate repressed the utilization of phthalates [8, 9]. Shorter chain compounds are metabolized at higher rates as compared to their longer chain counterparts [47]. The rates of anaerobic degradation are low as compared to aerobic degradation [8, 9, 53].

Aerobic metabolism

Aerobic degradation of phthalate isomers by bacteria was first reported by Evans in 1955 [29]. Presence of phthalates in the medium induces proteins that are involved in its transport and degradation [16, 27, 40, 87, 104]. Longer chain phthalate esters are difficult to degrade [102]. Using various metabolic studies like growth profiles, enzyme induction, whole-cell oxygen uptake and plate assay studies, the complete metabolic pathway for phthalate isomers and

their esters is deciphered [73, 84, 99, 104, 107] (Fig. 3). In this article we review the aerobic degradation of phthalate isomers and their esters in detail.

Phthalate esters are first hydrolyzed to their respective phthalate isomers by esterases. In *Gordonia* sp. strain MTCC 4818, esterase was shown to be inducible and had broad substrate specificity [14]. Some microbes can directly hydrolyze diesterified phthalate and terephthalate to respective phthalate isomers by *trans*-esterification [6, 58]. *Sphingomonas* sp. DEP-AD1 could grow by hydrolyzing diethyl phthalate, dibutyl phthalate and diethyl hexylphthalate; but could not metabolize aromatic ring of phthalate and benzoate suggesting that the organism utilizes aliphatic metabolites generated after de-esterification [30].

Phthalate isomers are transported inside the cell by specific permeases, which in turn induces specific phthalate isomer dioxygenases viz, phthalate-, isophthalate-, or terephthalate- dioxygenase. These enzymes catalyze the initial step of incorporation of two hydroxyl groups on the phthalate rings to yield respective phthalate dihydrodiols (Fig. 3). Based on the position of ring-hydroxylation, *o*-phthalate dioxygenase is either phthalate 4,5-dioxygenase, found mostly in Gram negative bacteria [26, 49]; or phthalate 3,4-dioxygenase, present mostly in Gram positive bacteria [35]. However, there are some exceptions where phthalate 3,4-dioxygenase is present in Gram negative organism like *Pseudomonas aeruginosa* sp. strain PP4 [99]. Action of phthalate 4,5-dioxygenase produces *cis*-4,5-dihydroxy-4,5-dihydrophthalate, which is dehydrogenated by *cis*-phthalate dihydrodiol dehydrogenase to 4,5-dihydroxyphthalate. By the action of 4,5-dihydroxyphthalate decarboxylase, this dihydroxylated phthalate undergoes decarboxylation to yield 3,4-dihydroxybenzoate [4,11,80]. Action of phthalate 3,4-dioxygenase produces *cis*-3,4-dihydroxy-3,4-dihydrophthalate, which is converted to 3,4-dihydroxyphthalate and subsequently decarboxylated to yield 3,4-dihydroxybenzoate by 3,4-dihydroxyphthalate decarboxylase [27] (Fig. 3).

Metabolism of terephthalate is initiated by double hydroxylation of the ring at positions 1 and 2, catalyzed by terephthalate 1,2-dioxygenase to yield 2-hydro-1,2-dihydroxy terephthalic acid, which is further metabolized to 3,4-dihydroxybenzoate [16, 88, 99, 104] (Fig. 3).

Compared to phthalate and terephthalate, isophthalate was found to be resistant to the microbial degradation as evident from the fact that very few microbial species have been reported to utilize isophthalate as the sole source of carbon and energy [49, 50, 99, 104]. Metabolic analysis suggest that isophthalate is double hydroxylated at position 3 and 4 by isophthalate 3,4-dioxygenase to yield 4-hydro-

Table 1 Some representative examples of bacteria that degrade phthalate isomers and their esters

Organism	Compounds metabolized	Intermediary metabolites	Ref
Anaerobic degradation			
<i>Pelotomaculum terephthalicum</i> , <i>Pelotomaculum isophthalicum</i>	Isophthalate, phthalate and terephthalate	Benzoate, acetate, methane	81
<i>Pseudomonas</i> sp. P136	Phthalate isomers	CoA esters of phthalate, benzoate, cyclohex-1-ene-carboxylate, 2-oxocyclohexane carboxylate, pimelate, acetate	74
<i>Pelotomaculum thermopropionicum</i> , <i>Desulfotomaculum thermobenzoicum</i> , <i>Desulfotomaculum nigrificans</i> , <i>Desulfotomaculum thermosapovorans</i> , <i>Syntrophobacter fumaroxidans</i> , <i>Syntrophus gentianae</i> , <i>Desulfovibrio vulgaris</i> , <i>Methanospirillum hungatei</i> , <i>Methanosaeta concilii</i>	Phthalate isomers	Benzoate, acetate	82
Uncharacterized microorganisms in sludge	Benzoate, phthalate isomers, dimethyl phthalate, dimethyl terephthalate	Monomethyl terephthalate, terephthalate, monomethyl phthalate, phthalate, esters of CoA	53
Uncharacterized microorganisms in sludge	Di- <i>n</i> -butyl phthalate, diethyl phthalate, dimethyl phthalate, butylbenzyl phthalate	Monophthalate ester, phthalate, benzoate, methane	89
Uncharacterized microorganisms in sludge	Dimethyl phthalate, di- <i>n</i> -butyl phthalate, di- <i>n</i> -octyl phthalate	Methane	47
Aerobic Degradation			
<i>Pseudomonas aureofaciens</i> , <i>Pseudomonas fluorescens</i> , <i>Sphingomonas paucimobilis</i> , <i>Xanthomonas maltophilia</i>	Phthalate and Dimethyl phthalate	Monomethyl phthalate	102
<i>Rhodococcus</i> sp. strain DK17	Phthalate, Terephthalate	3,4-Dihydroxyphthalate, 3,4-dihydroxybenzoate	16
<i>Gordonia</i> sp. strain MTCC 4818	Butyl benzyl phthalate, Di- <i>n</i> -butyl phthalate, Diisobutyl phthalate, Diphenyl phthalate	Mono- <i>n</i> -butyl phthalate, monobenzyl phthalate, phthalate	14
<i>Sphingomonas</i> sp. DK4, <i>Corynebacterium</i> sp O18	Diethylphthalate, Dipropylphthalate, Di- <i>n</i> -butylphthalate, Benzylbutylphthalate, Diphenylphthalate, Dicyclohexyl phthalate, Dihexyl phthalate, Di-(2-ethylhexyl)phthalate		10
<i>Sphingomonas yanoikuyae</i> DOS01, <i>Variovorax paradoxus</i> T4	Dimethyl terephthalate	Monomethyl terephthalate, terephthalate	103
<i>Closterium lunula</i>	Dimethyl phthalate	Phthalate	107
<i>Pasteurella multocida</i> Sa	Dimethyl terephthalate	Monomethyl terephthalate, monoethyl terephthalate, terephthalate	58
<i>Delftia</i> sp. TBKNP-05	Di- <i>n</i> -butylphthalate	Monobutyl phthalate, phthalate, 4,5-dihydroxy phthalate, 3,4-dihydroxybenzoate	78
<i>Arthrobacter Keyseri</i> 12B	Dibutyl phthalate, Phthalate	Phthalate, 3,4-dihydroxyphthalate, 3,4-dihydroxybenzoate	26

Table 1 (Continued)

Organism	Compounds metabolized	Intermediary metabolites	Ref
<i>Bacillus subtilis</i> No. 66	Di-2-ethylhexyl phthalate, Dibutyl phthalate	Monobutyl phthalate, mono-2-ethyl phthalate, phthalate, 3,4-dihydroxybenzoate	83
<i>Comamonas testosteroni</i> YZW-D	Isophthalate, Terephthalate	3,4-dihydroxybenzoate	104
<i>Mycobacterium vanbaalenii</i> PYR1	Phthalate	3,4-dihydroxyphthalate, 3,4-dihydroxybenzoate	91
<i>Moraxella</i> sp. VG45	Phthalate	4,5-dihydroxyphthalate, 3,4-dihydroxybenzoate	84
<i>Micrococcus</i> sp. 12B	Dibutyl phthalate, Phthalate	Monobutyl phthalate, phthalate, 3,4-dihydroxyphthalate, 3,4-dihydroxybenzoate	27
<i>Burkholderia cepacia</i> DBO1	Phthalate	4,5-dihydroxyphthalate, 3,4-dihydroxybenzoate	12

Fig. 2 Proposed anaerobic degradation pathway of phthalate esters. Few key enzymes of the metabolic pathway are: a, CoA ligase; b, decarboxylase; c, cyclo-1-ene-1-carboxyl-CoA hydratase; d, 2-hydroxycyclohexane-1-carboxyl-CoA dehydrogenase; e, 2-ketocyclohexane-1-carboxyl-CoA hydrolase.

Fig. 3 Metabolic pathway of phthalate esters. Few key enzymes of the metabolic pathway are: a, esterase; b, isophthalate 3,4-dioxygenase; c, phthalate 3,4-dioxygenase; d, phthalate 4,5-dioxygenase; e, terephthalate 1,2-dioxygenase; f, protocatechuate 4,5-dioxygenase; g, protocatechuate 3,4-dioxygenase.

3,4-dihydroxyisophthalate, which is further decarboxylated to 3,4-dihydroxybenzoate [99, 104] (Fig. 3).

All phthalate isomer metabolic pathways has 3,4-dihydroxybenzoate as a central metabolic intermediate, which is further ring-cleaved either by protocatechuate 4,5-dioxygenase (referred as extradiol, *meta* pathway) or protocatechuate 3,4-dioxygenase (referred as intradiol, *ortho* pathway) (Fig. 3). Protocatechuate 4,5-dioxygenase catalyzes the conversion of 3,4-dihydroxybenzoate to 4-carboxy-2-hydroxy-muconic semialdehyde, which is subsequently oxidized to pyruvate and oxaloacetate via 4-oxalocitramalate [26, 27, 35]. Protocatechuate 3,4-dioxygenase ring-cleaves 3,4-dihydroxybenzoate to yield β -carboxy-*cis,cis*-muconic acid [35, 99], which is further oxidized to succinyl-CoA and acetyl-CoA via β -ketoadipate [35, 111, 112].

The metabolic steps and the enzymes responsible for the conversion of phthalate esters to 3,4-dihydroxybenzoate constitutes the 'upper pathway', while the steps responsible for the conversion of 3,4-dihydroxybenzoate to central carbon pathway, are referred as the 'lower pathway'. The metabolic diversity in microorganisms is mostly observed in the lower pathway. In *A. keyseri* 12B, 3,4-dihydroxybenzoate is metabolized by both, 4,5-extradiol and 3,4-intradiol ring-cleavage [26]. *Rhodococcus* sp. strain RHA1 has two pathways for the degradation of 3,4-dihydroxybenzoate. Besides ring-cleavage, 3,4-dihydroxybenzoate is decarboxylated to catechol, which subsequently undergoes *ortho* ring-cleavage to *cis,cis*-muconate by catechol 1,2-dioxygenase [40]. In several bacterial species *o*-phthalate was shown to be an intermediate in phenanthrene metabolism [79].

Pseudomonas sp. strain PPD also metabolizes phenanthrene via phthalate [23]. Phenanthrene grown PPD cells did not show O₂ uptake on phthalate and 3,4-DHB, while phthalate grown cells showed considerable O₂ uptake on both phthalate and 3,4-DHB. This could be due to induction of permease when cells are grown on phthalate and not on phenanthrene. The cell-free extracts of phthalate grown cells showed enzyme activities of protocatechuate 3, 4-dioxygenase while phenanthrene grown cells showed protocatechuate 4,5-dioxygenase activity (unpublished data), suggesting that *Pseudomonas* sp. strain PPD has diverse pathway for phthalate metabolism.

Among reported phthalate degraders, very few strains like *Pseudomonas testosteroni* EN 5a [49], bacterial strain B and E [104], *Pseudomonas aeruginosa* strain PP4 [99], *Pseudomonas* strain PPD [99] and *Comamonas testosteroni* strain E6 [87], has the ability to degrade all three phthalate isomers as the carbon source. In these strains, metabolic pathways of respective phthalate isomers are initiated by specific ring-hydroxylating dioxygenase to yield respective diols which are subsequently metabolized and converged to 3,4-dihydroxybenzoate and further metabolized by either *ortho* or *meta* pathway to the central carbon cycle intermediates (Fig. 3).

Degradation by microbial consortia

Ability of organisms to degrade one or two specific phthalate isomer(s) suggest the probable lack of genetic material that code for degradative enzymes of other isomer(s). Hence, bacterial cocktail consisting of organisms specific and efficient for various isomers are preferred for effective bioremediation. For example, mixed culture of *Pseudomonas* and *Bacillus* metabolizes terephthalate at high concentrations (8 g/l) from waste waters completely as compared to partial utilization by a single strain [50]. Herada and Koiwa enriched *Alcaligenes*, *Arthrobacter* and *Corynebacterium* strains using three isomers of phthalate as carbon source. As a single isolate, none of them could grow on mixture of three isomers, however as mixed culture they could degrade all isomers efficiently [35]. Bacterial consortia mineralizes low molecular weight phthalate esters faster than higher molecular weight counterparts [94]. Consortia of *Sphingomonas* sp. DK4 and *Corynebacterium* sp. O18 have ability to degrade 8 different phthalate esters, however degradation efficiency enhanced when both strains were present simultaneously [10]. *Pseudomonas fluorescens*, *Pseudomonas aureofaciens* and *Sphingomonas paucimobilis* as consortia can hydrolyze dimethyl phthalate to phthalate *via* monomethyl phthalate, which is further metabolized

as the carbon source. As single isolate they failed to utilize it as the carbon source [102].

Enzymology

Metabolism of phthalate isomers and esters involves the participation of a plethora of proteins that include the transport of phthalates by specific permeases; de-esterification by esterases or hydrolases; ring-hydroxylating dioxygenases that hydroxylates the phthalate ring; and subsequently the ring-cleaving dioxygenases to yield aliphatic metabolites. Most of the enzymes of both the upper and lower pathways, involved in the metabolism of phthalate isomers and their esters, are inducible.

Esterase/Hydrolase

The initial step in the metabolism of phthalate esters is hydrolysis of ester bond by esterase/hydrolase to yield monoesters and then phthalate isomer [49, 66, 71, 78, 83, 100]. Esterases/hydrolase are inducible and show broad substrate specificity [10, 14, 78, 83, 102], while few are specific [103]. The enzyme is either monomeric (molecular weight, 56 kDa) or dimeric (monomer molecular weight, 31 kDa or 27 kDa) [1, 72]. The hydrolase from *Gordonia* species strain P8219 contained a pentapeptide motif GX SXG, a conserved sequence of serine hydrolase [72].

Permeases

The permeases are responsible for the transport of phthalate isomers across the membrane specifically. They belong to the major facilitator superfamily of transport proteins with 12 hydrophobic membrane-spanning α -helices. Permease from *B. cepacia* ATCC 17616 and *P. putida* NMH102-2 showed close similarity with anion:cation symporter family while permease from *B. cepacia* Pc701 showed similarity with metabolite:H⁺ symporter family [12]. Permeases belong to the catabolic operon and reported to have multiple genes [12, 26, 87, 104].

Phthalate dioxygenase

The key step in the degradation of the phthalate isomer is the hydroxylation of aromatic ring by ring-hydroxylating dioxygenase. Of the reported dioxygenases, phthalate dioxygenase is a well characterized enzyme. The enzyme has been purified and found to be a multi-component

Fig. 4 Schematic representation of an electron transfer in phthalate dioxygenases. Phthalate dioxygenase reductase (PDR) and phthalate dioxygenase oxygenase components (PDO) with their constituents of electron transfer [62].

system with mini electron transport chain (Fig. 4). Phthalate 4,5-dioxygenase (EC No. 1.14.12.7) from *Burkholderia cepacia* DB01 (earlier called *Pseudomonas cepacia*) has two components: i) reductase (PDR), a monomeric 34 kDa flavo-iron-sulfur protein containing FMN and plant-ferridoxin-type [2Fe-2S] center, which requires NADH for its activity. The hydride transfer from NADH reduces the FMN, with the subsequent movement of electrons to [2Fe-2S] center that is present in the C-terminal domain of the protein. The Fe atoms are bound by cysteines from the loop sequence $^{272}\text{C-E-S-G-T-C-G-S-C}$ and ^{308}Cys and; ii) oxygenase (PDO), native molecular weight ~217 kDa, a homotetramer with subunit molecular weight of 48 kDa. Oxygenase component possess a non-heme iron protein with one Rieske-type [2Fe-2S] center and one mononuclear iron, which are responsible for double hydroxylation of

the phthalate ring [4, 18, 33]. Compared to phthalate, oxygenase component showed 65–70 % activity on 2,3- and 3,4-dicarboxypyridine, and 4-chloro-*o*-phthalate. The enzyme has K_m 0.3 and 0.125 mM for phthalate and oxygen, respectively [3]. Of the two components, the structure of reductase component is resolved at 2 Å [18, 62]. Based on recent biophysical and modeling studies it has been proposed that phthalate 4,5-dioxygenase has two planar rings of $\alpha_3-\alpha_3$ arrangement one above the other unlike other Rieske dioxygenases that has α and β subunits [96].

Phthalate 3,4-dioxygenase from Gram positive strains possesses large and small oxygenase subunits including ferridoxin and ferridoxin reductase subunits [16, 26, 91]. Exceptionally the enzyme from *R. erythropolis* sp. S1 is a homo-tetramer with subunit molecular weight of 56 kDa. Enzyme is a flavoprotein containing FAD and requires

NADH for activity. The enzyme might be a dual function enzyme with both oxygenase and reductase activities [92, 93].

Terephthalate- and isophthalate- dioxygenase

Like *o*-phthalate dioxygenase, terephthalate 1,2-dioxygenase (EC No. 1.14.12.15) also has both reductase and oxygenase components [88,90]. The oxygenase component from *Delftia tsuruhatensis* strain T7 is $\alpha_3\beta_3$ (native molecular weight 235 kDa) with subunits of molecular weight 46 and 17 kDa for α and β , respectively. In *Comamonas testosteroni* T2, oxygenase component (native molecular weight of 126 kDa) constitutes α and β subunits with molecular masses of 49 and 18 kDa, respectively. The oxygenase component in these strains contained Rieske centre [2Fe-2S] and mononuclear Fe(II) binding sites [88, 90]. Very little is known about isophthalate dioxygenase. The enzyme from *P. aeruginosa* strain PP4, *Pseudomonas* strain PPD and *Acinetobacter lwoffii* strain ISP4 was found to be inducible, had both oxygenase and reductase components, requires NADH, act specifically on isophthalate [99].

Protocatechuate dioxygenase

This is the key ring-cleaving dioxygenase, breaks the aromaticity and generate aliphatic intermediate which enters the central carbon cycle. Based on the position of ring-cleavage the enzyme can be protocatechuate 3,4-dioxygenase (*ortho*, EC. No. 1.13.11.3) or protocatechuate 4,5-dioxygenase (*meta*, EC. No. 1.13.11.8). Both these enzymes are well characterized and found to be heterodimers with α and β subunits and requires Fe^{+2} for their activity [32, 64, 67, 76, 104, 109, 111, 112]. Some of them are reported to be octamers or decamers [5, 25, 105, 108]. In *Pseudomonas aeruginosa*, protocatechuate 3,4-dioxygenase is a octamer, each subunit of octamer has two α and two β subunits with molecular weight of 22.5 and 25 kD, respectively. Interestingly both the subunits contain proline at the N-terminal region [108]. Crystal structures of both protocatechuate 3,4- and 4,5-dioxygenase has been solved at 2.1 to 2.2 Å [32, 75, 95, 101]. In protocatechuate 4,5-dioxygenase the non-heme iron of the active site is coordinated by His12, His61, Glu242 and a water molecule in a distorted trigonal pyramidal geometry [95] while in protocatechuate 3,4-dioxygenase it is coordinated by Tyr447, His460, Tyr408, His462 of β subunits, and a hydroxide ion in a distorted bipyramidal geometry. The substrate binding pocket is ~5 Å deep and covered by two helices and one β strand. Two

helices from the α -subunit forms a lid and prevents the escape of substrate [95]. In protocatechuate 3,4-dioxygenase the substrate has direct access into the active site through a funnel shaped opening. Different amino acids of the funnel make the channel narrow as the substrate gets into the active site. The funnel has a dimensions of 18 Å × 10 Å at the top and 5 Å × 7 Å in the active site cavity [101].

Genetics of phthalate degradation

Genes involved in the metabolism of phthalate isomers are present either on plasmids, chromosome, insertion elements, or both on plasmid and chromosome [11, 15, 26, 39, 84, 87, 91, 112]. Compared to terephthalate and isophthalate, phthalate degrading genes are well characterized with respect to their organization and regulation. Genes are organized in operon(s). The genes and their organization from various organisms is summarized in Table 2 and Fig. 5.

In *Burkholderia cepacia* DBO1, five structural genes coding for degradation of phthalate to protocatechuate are located on chromosome. These genes are arranged in three transcriptional units based on orientation: *ophA1*, *ophDC*, and *ophA2B*. The two genes (*ophA1* and *ophA2*) encoding the oxygenase and reductase components of phthalate dioxygenase, are located at the opposite ends, approximately 7.0 kb apart (Table 2, Fig. 5). Similar genetic arrangement is seen in *B. cepacia* strain ATCC 17616 [11]. *ophD* is a putative phthalate transporter [12]. Phthalate dioxygenases can also hydroxylate quinolinate, a substrate mimic of phthalate and an intermediate in the biosynthesis of NAD^+ . Strain *B. cepacia*, hence recruited an extra copy of quinolinate phosphoribosyl transferase (*ophE*) in the phthalate catabolic genes to improve the NAD^+ biosynthesis which otherwise would have been rate limiting for the growth [13].

In *Mycobacterium vanbaalenii* PYR-1 the putative phthalate (*pht*) operon is located on genome and contain putative regulatory protein gene, *phtR*, transcribed from the opposite strand and is upstream of the operon [91]. The operon consists of *phtAa*, *phtAb*, *phtU* (an unknown ORF), *phtB*, *phtAc* and *phtAd* and no decarboxylase gene (Table 2, Fig. 5). The operon gene products share 53–78% identity and 66– 88% similarity with their counterparts from *Terrabacter* sp. DBF63 and *A. keyseri* 12B, however they differ in the placement and orientation of the regulatory gene.

In *Arthrobacter keyseri*, 26.3 kbp of the plasmid (pRE1-130 kbp) harbors genes encoding proteins for transport, regulation and degradation of phthalate. The 8.14 kbp fragment of 26.3 kbp has the complete phthalate genes from phthalate till protocatechuate called *pht* operon. The genes being *phtAa*, *phtAb*, *phtAc*, *phtAd*, *phtB*, *phtC*, *phtR*

Table 2 Genes involved in the degradation of phthalate isomers and their esters from different organisms.

Organism	Location (Size of operon)	Gene (bp)	Protein	Ref
<i>Burkholderia cepacia</i> strain DBO1	Genome	<i>ophA1</i> (969)	Phthalate dioxygenase reductase	11
		<i>orf1</i> (351)	Putative phthalate permease N terminal	
		<i>ophD</i> (999)	Putative phthalate permease C terminal	
		<i>ophC</i> (993)	4,5-Dihydroxyphthalate decarboxylase	
		<i>orf2</i> (597)	Putative transcriptional regulator	
		<i>ophE</i> (846)	Quinolinate phosphoribosyl transferase	
		<i>ophB</i> (1176)	Phthalate dihydrodiol dehydrogenase	
		<i>ophA2</i> (1332)	Phthalate dioxygenase	
<i>Mycobacterium vanbaalenii</i> PYR-1	Genome (6.7 kb)	<i>phtR</i> (805)	Operon regulator	91
		<i>phtAa</i> (1464)	Phthalate dioxygenase large subunit	
		<i>phtAb</i> (600)	Phthalate dioxygenase small subunit	
		<i>phtU</i> (306)	Unknown protein	
		<i>phtB</i> (804)	Dihydrodiol dehydrogenase	
		<i>phtAc</i> (201)	Phthalate dioxygenase ferridoxin subunit	
		<i>phtAd</i> (1242)	Phthalate dioxygenase ferredoxin reductase	
<i>Arthrobacter keyseri</i> 12B	Plasmid (26.3 kb)	<i>ptrD</i> (663)	Transporter, substrate binding protein	26
		<i>ptrA</i> (828)	Transporter, ATPase	
		<i>ptrB</i> (801)	Transporter, permease 1	
		<i>ptrC</i> (795)	Transporter, permease 2	
		<i>pehA</i> (657)	Putative phthalate ester hydrolase	
		<i>phtB</i> (864)	3,4-Dihydroxy-3,4-dihydrophthalate dehydrogenase	
		<i>phtAa</i> (1421)	Phthalate dioxygenase, large subunit	
		<i>phtAb</i> (600)	Phthalate dioxygenase, small subunit	
		<i>phtAc</i> (195)	Phthalate dioxygenase, Ferridoxin	
		<i>phtAd</i> (1212)	Phthalate dioxygenase, Ferridoxin reductase	
		<i>phtC</i> (747)	3,4-Dihydroxyphthalate decarboxylase	
		<i>phtR</i> (792)	<i>pht</i> operon regulator	
<i>Rhodococcus</i> strain DK17	Plasmids (18 kb)	<i>ophC</i> (732)	3,4-Dihydroxyphthalate decarboxylase	16
		<i>ophA4</i> (1242)	Phthalate 3,4-dioxygenase reductase	
		<i>ophA3</i> (195)	Phthalate 3,4-dioxygenase ferridoxin	
		<i>ophB</i> (807)	Phthalate dihydrodiol dehydrogenase	
		<i>orf0</i> (12)	Unknown protein	
		<i>ophA2</i> (627)	Phthalate 3,4-dioxygenase, oxygenase small subunit	
		<i>ophA1</i> (1446)	Phthalate 3,4-dioxygenase, oxygenase large subunit	
		<i>ophR</i> (807)	Phthalate operon regulator	
		<i>pehA</i> (666)	Putative phthalate ester hydrolase	
		<i>ptrC</i> (816)	Transporter permease 2	
		<i>ptrB</i> (810)	Transporter permease 1	
		<i>ptrA</i> (813)	Transporter ATPase	

Table 2 (Continued)

Organism	Location (Size of operon)	Gene (bp)	Protein	Ref
<i>Terrabacter</i> strain DBF63	Plasmid (14 kb)	<i>ptrD</i> (678)	Substrate binding transporter	39
		<i>tphR</i> (807)	Terephthalate operon regulator	
		<i>tphA1</i> (1266)	Terephthalate oxygenase large subunit	
		<i>tphA2</i> (471)	Terephthalate oxygenase small subunit	
		<i>tphB</i> (936)	Terephthalate dihydrodiol dehydrogenase	
		<i>tphA4</i> (1011)	Terephthalate dioxygenase reductase	
<i>Comamonas</i> strain E6	Genome (7.3 kb)	ORF14 (1263)	Transposase	87
		<i>phtA1</i> (1479)	Phthalate dioxygenase large subunit	
		<i>phtA2</i> (594)	Phthalate dioxygenase small subunit	
		<i>phtB</i> (810)	<i>cis</i> -Dihydrodiol dehydrogenase	
		<i>phtA3</i> (195)	Ferredoxin	
		<i>phtA4</i> (1239)	Ferredoxin reductase	
		<i>phtC</i> (720)	Decarboxylase	
		<i>phtR</i> (795)	Regulatory protein	
	Genome (11 kb)	<i>tphR_I</i> (767)	Ic1R –type transcriptional regulator	
		<i>tphC_I</i> (968)	Periplasmic terephthalate binding receptor	
		<i>tphA2_I</i> (1241)	Terephthalate dioxygenase-oxygenase large subunit	
		<i>tphA3_I</i> (464)	Terephthalate dioxygenase-oxygenase small subunit	
		<i>tphB_I</i> (947)	Dehydrogenase	
		<i>tphA1_I</i> (1010)	Reductase component	
		<i>tphR_{II}</i> (767)	Ic1R –type transcriptional regulator	
<i>Comamonas testosteroni</i> strain YZW-D	Genome (18 kb)	<i>tphC_{II}</i> (968)	Periplasmic terephthalate binding receptor	104
		<i>tphA2_{II}</i> (1241)	Terephthalate dioxygenase-oxygenase large subunit	
		<i>tphA3_{II}</i> (464)	Terephthalate dioxygenase-oxygenase small subunit	
		<i>tphB_{II}</i> (947)	Dehydrogenase	
		<i>tphA1_{II}</i> (1010)	Reductase component	
		<i>tphR</i> (789)	Regulator protein	
		<i>tphC</i> (969)	Permease	
		<i>tphA2</i> (1242)	Terephthalate dioxygenase large subunit	
		<i>tphA3</i> (465)	Terephthalate dioxygenase small subunit	
		<i>tphB</i> (948)	Terephthalate dihydrodiol dehydrogenase	
		<i>tphA1</i> (1011)	Terephthalate dioxygenase reductase	
		<i>iphA2</i> (1275)	Isophthalate dioxygenase oxygenase	
		<i>iphC</i> (990)	Permease	
		<i>iphB</i> (780)	Isophthalate 3,4-dihydrodiol dehydrogenase	
		<i>iphA1</i> (978)	Isophthalate dioxygenase reductase	

(See Table 2 and Fig. 5). Operon *pcmDECABF* codes for the enzymes involved in the metabolism of protocatechuate to pyruvate and oxaloacetate. Enzyme induction studies suggests that: (i) a constitutive phthalate ester hydrolase

(esterase); (ii) phthalate 3,4-dioxygenase and 3,4-dihydroxy-3,4-dihydrophthalate dehydrogenase, inducible by phthalate; (iii) 3,4-dihydroxyphthalate decarboxylase, constitutive but also slightly inducible by phthalate; and

Fig. 5 Structural organization of phthalate, terephthalate and isophthalate degrading genes from various organisms. The size of genes is not to the scale. The arrows indicate the direction of transcription. The enzymes coded by respective genes are organized as operon(s) either on plasmid or on chromosome, are given in Table 2.

(iv) protocatechuate *metacleaveage* pathway, inducible by protocatechuate [26].

In the *Rhodococcus* strain DK 17 catabolic genes for phthalate and terephthalate metabolism are arranged in two different operons separated by 6.7 kb fragment. The genes are oriented in opposite direction [16]. The putative phthalate operon contains the genes *ophA1*, *ophA2*, *orf0*, *ophB*, *ophA3*, *ophA4* and *ophC* (Table 2 and Fig. 5). Operon for terephthalate degradation contains *tphA1*, *tphA2* located approximately 6.7 kb away from the *ophA1* gene and are transcribed in opposite directions. A gene encoding a terephthalate dihydrodiol dehydrogenase (*tphB*) is located immediately after *tphA1A2*, and the ferredoxin reductase gene (*tphA4*) follows. Both the operons contain regulatory proteins oriented in opposite directions. The strain contains

3 different plasmids pDK1, pDK2 and pDK3 with sizes of 380, 330 and 750 kb respectively [16]. The phthalate and terephthalate genes are duplicated on both pDK2 and pDK3 plasmids. The dihydroxyphthalate decarboxylase genes of both the plasmids are simultaneously expressed and hence the strain takes advantage due to gene dosage [15].

In *Terrabacter* sp. strain DBF63 the 7 *pht* genes, *phtA1*, *phtA2*, *phtB*, *phtA3*, *phtA4*, *phtC* and *phtR* are arranged adjacent to the angular dioxygenases gene (Table 2 and Fig. 5). The genes are part of insertional sequence ISTesp2, which was also identified in another *Terrabacter* strain DPO360 [39].

Comamonas sp. strain E6, grows on all the three isomers of phthalate, two identical clusters of terephthalate degrading genes are found that code for transcriptional regulator,

permease, terephthalate dioxygenases, reductase and dehydrogenase. These are converged at 3,4-DHB that undergoes meta cleavage to enter kreb's cycle [87]. The genetic arrangement is seen in Fig. 5.

The genetic makeup of isophthalate degradation are reported from *Comamonas testosteroni* YZW-D, which also degrades terephthalate and isophthalate. The genes for the degradation are arranged in two separate operons and are induced specifically by the respective phthalate isomer. The genes code for putative isophthalate dioxygenases, reductase, dehydrogenase, a regulator and transporter protein [104] (Table 2 and Fig. 5). Unusually the dioxygenases and reductase component are arranged at the extremes of the operon. The isomers converge at 3,4-DHB which undergoes meta cleavage.

The ability of organisms to degrade *o*-phthalate, terephthalate and their esters is known for past few decades. However, very little information is available regarding degradation of isophthalate and its genetic regulation. Even though the phthalates are well known for their metabolic inhibition and estrogenic activities, microbes are evolving in nature to overcome these effects and successfully metabolize phthalate isomers and esters. The most valuable organism will be the one which has the ability to degrade all phthalate isomers effectively as the carbon and energy source. The understanding of metabolic pathways, enzymes involved and gene regulation of such strain will enable to genetically modify strains for effective bioremediation and environmental clean up.

Acknowledgement

PP would like to thank DBT and BRNS, Govt of India for the research grant. VKC would like to thank CSIR, Govt. of India. Thanks to Prabin D Majhi and Jaigeeth D for critical reading of the MS.

References

1. Akita K, Naitou C and Maruyama K (2001) Purification and characterization of an esterase from *Micrococcus* sp. YGJ1 hydrolyzing phthalate esters. *Biosci Biotechnol Biochem* 65: 1680–1683
2. Autian J (1973) Toxicity and health threats of phthalate esters: review of the literature *Environ Health Perspect* 4:3–26
3. Batie CJ and Ballou DP (1990) Phthalate dioxygenase Methods. *Enzymol* 188:61–70
4. Batie CJ, LaHaie E and Ballou DP (1987) Purification and characterization of phthalate oxygenase and phthalate oxygenase reductase from *Pseudomonas cepacia*. *J Biol Chem* 262:1510–1518
5. Bull C and Ballou DP (1981) Purification and properties of protocatechuate 3,4-dioxygenase from *Pseudomonas putida*. A new iron to subunit stoichiometry. *J Biol Chem* 256:12673–12680
6. Cartwright CD, Owen SA, Thompson IP and Burns RG (2000) Biodegradation of diethyl phthalate in soil by a novel pathway FEMS. *Microbiol Lett* 186:27–34
7. Caughey WS, Hellerman L and Smiley JD (1957) L-glutamic acid dehydrogenase; structural requirements for substrate competition; effect of thyroxine. *J Biol Chem* 224:591–607
8. Chang BV, Liao CS and Yuan SY (2005) Anaerobic degradation of diethyl phthalate, di-*n*-butyl phthalate, and di-(2-ethylhexyl) phthalate from river sediment in Taiwan. *Chemosphere* 58:1601–1607
9. Chang BV, Liao GS and Yuan SY (2005) Anaerobic degradation of di-*n*-butyl phthalate and di-(2-ethylhexyl) phthalate in sludge. *Bull Environ Contam Toxicol* 75:775–782
10. Chang BV, Yang CM, Cheng CH and Yuan SY (2004) Biodegradation of phthalate esters by two bacteria strains. *Chemosphere* 55:533–538
11. Chang HK and Zylstra GJ (1998) Novel organization of the genes for phthalate degradation from *Burkholderia cepacia* DBO1. *J Bacteriol* 180:6529–6537
12. Chang HK and Zylstra GJ (1999) Characterization of the phthalate permease OphD from *Burkholderia cepacia* ATCC 17616. *J Bacteriol* 181:6197–6199
13. Chang HK and Zylstra GJ (1999) Role of quinolinate phosphoribosyl transferase in degradation of phthalate by *Burkholderia cepacia*. DBO1 *J Bacteriol* 181:3069–3075
14. Chatterjee S and Dutta TK (2003) Metabolism of butyl benzyl phthalate by *Gordonia* sp. strain MTCC 4818. *Biochem Biophys Res Commun* 309:36–43
15. Choi KY, Kim D, Chae JC, Zylstra GJ and Kim E (2007) Requirement of duplicated operons for maximal metabolism of phthalate by *Rhodococcus* sp. strain DK17 *Biochem Biophys Res Commun* 357:766–771
16. Choi KY, Kim D, Sul WJ, Chae JC, Zylstra GJ, Kim YM and Kim E (2005) Molecular and biochemical analysis of phthalate and terephthalate degradation by *Rhodococcus* sp. strain DK17. *FEMS Microbiol Lett* 252:207–213
17. Colborn T, vom Saal FS and Soto AM (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* 101:378–384
18. Correll CC, Batie CJ, Ballou DP and Ludwig ML (1992) Phthalate dioxygenase reductase: a modular structure for electron transfer from pyridine nucleotides to [2Fe-2S]. *Science* 258:1604–1610
19. Cunliffe D, Leason M, Parkin D and Lea PJ (1983) The inhibition of glutamate dehydrogenase by derivatives of isophthalic acid. *Phytochemistry* 22:1357–1360
20. Dai G, Cui L, Song L, Cheng J, Zhong Y, Zhao R and Wang X (2005) Bladder epithelial cell proliferation of rats induced by terephthalic acid-calcium. *Food Chem Toxicol* 43: 217–224
21. Dai GD, Cui LB, Song L, Zhao RZ, Cheng JF, Liu MX, Zhou JW, Xiao H and Wang XR (2005) Induction of bladder lesion by terephthalic acid and its mechanism. *Biomed Environ Sci* 18:211–219
22. Den HE and Schoeters G (2006) Endocrine disrupters and human puberty. *Int J Androl* 29:264–271

23. Deveryshetty J, Suvekbala V, Varadamshetty G and Phale PS (2007) Metabolism of 2-, 3- and 4-hydroxybenzoates by soil isolates *Alcaligenes* sp. strain PPH and *Pseudomonas* sp. strain PPD. *FEMS Microbiol Lett* 268:59–66
24. Dillingham EO and Autian J (1973) Teratogenicity, mutagenicity, and cellular toxicity of phthalate esters. *Environ Health Perspect* 3:81–89
25. Durham DR, Stirling LA, Ornston LN and Perry JJ (1980) Intergeneric evolutionary homology revealed by the study of protocatechuate 3,4-dioxygenase from *Azotobacter vinelandii*. *Biochemistry* 19:149–155
26. Eaton RW (2001) Plasmid-encoded phthalate catabolic pathway in *Arthrobacter keyseri* 12B. *J Bacteriol* 183: 3689–3703
27. Eaton RW and Ribbons DW (1982) Metabolism of dimethylphthalate by *Micrococcus* sp. strain 12B. *J Bacteriol* 151: 465–467
28. Ejlertsson J and Svensson BH (1996) Degradation of bis(2-ethylhexyl) phthalate constituents under methanogenic conditions. *Biodegradation* 7:501–506
29. Evans WC (1955) The early intermediate formed in the oxidative metabolism of phthalic acid by certain soil bacteria. *Biochem J* 61:x
30. Fang HH, Liang D and Zhang T (2007) Aerobic degradation of diethyl phthalate by *Sphingomonas* sp. *Bioresour Technol* 98:717–720
31. Fisher JS (2004) Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome *Reproduction* 127:305–315
32. Fujisawa H and Hayaishi O (1968) Protocatechuate 3,4-dioxygenase. I. Crystallization and characterization. *J Biol Chem* 243:2673–2681
33. Gassner GT and Ballou DP (1995) Preparation and characterization of a truncated form of phthalate dioxygenase reductase that lacks an iron-sulfur domain *Biochemistry* 34: 13460–13471
34. Gesler RM (1973) Toxicology of di-2-ethylhexyl phthalate and other phthalic acid ester plasticizers. *Environ Health Perspect* 3:73–79
35. Gibson DT and Subramanian V (1984) Microbial degradation of aromatic hydrocarbons. In: *Microbial degradation of organic compounds*, (Gibson DT ed), Marcel Dekker, New York, pp 181–252
36. Gray LE, Jr., Ostby J, Furr J, Price M, Veeramachaneni DN and Parks L (2000) Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicol Sci* 58:350–365
37. Grifoll M, Selifonov SA and Chapman PJ (1994) Evidence for a novel pathway in the degradation of fluorene by *Pseudomonas* sp. strain F274. *Appl Environ Microbiol* 60: 2438–2449
38. Gross FC and Colony JA (1973) The ubiquitous nature and objectionable characteristics of phthalate esters in aerospace technology. *Environ Health Perspect* 3:37–48
39. Habe H, Miyakoshi M, Chung J, Kasuga K, Yoshida T, Nojiri H and Omori T (2003) Phthalate catabolic gene cluster is linked to the angular dioxygenase gene in *Terrabacter* sp. strain DBF63. *Appl Microbiol Biotechnol* 61:44–54
40. Hara H, Eltis LD, Davies JE and Mohn WW (2007) Transcriptomic analysis reveals a bifurcated terephthalate degradation pathway in *Rhodococcus* sp. strain RHA1. *J Bacteriol* 189:1641–1647
41. Hauser R, Meeker JD, Singh NP, Silva MJ, Ryan L, Duty S and Calafat AM (2007) DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites. *Hum Reprod* 22:688–695
42. Heitkamp MA, Freeman JP, Miller DW and Cerniglia CE (1988) Pyrene degradation by a *Mycobacterium* sp.: identification of ring oxidation and ring fission products *Appl Environ Microbiol.* 54:2556–2565
43. Hudson RC and Daniel RM (1993) L-glutamate dehydrogenases: distribution, properties and mechanism. *Comp Biochem Physiol B* 106:767–792
44. Ito R, Seshimo F, Haishima Y, Hasegawa C, Isama K, Yamagami T, Nakahashi K, Yamazaki H, Inoue K, Yoshimura Y, Saito K, Tsuchiya T and Nakazawa H (2005) Reducing the migration of di-2-ethylhexyl phthalate from polyvinyl chloride medical devices. *Int J Pharm* 303:104–112
45. Jaeger RJ and Rubin RJ (1973) Extraction, localization, and metabolism of di-2-ethylhexyl phthalate from PVC plastic medical devices. *Environ Health Perspect* 3:95–102
46. Jaeger RJ and Rubin RJ (1970) Plasticizers from plastic devices extraction, metabolism, and accumulation by biological systems. *Science* 170:460–462
47. Jianlong W, Lujun C, Hanchang S and Yi Q (2000) Microbial degradation of phthalic acid esters under anaerobic digestion of sludge. *Chemosphere* 41:1245–1248
48. Kelley I, Freeman JP, Evans FE and Cerniglia CE (1993) Identification of metabolites from the degradation of fluoranthene by *Mycobacterium* sp. strain PYR-1. *Appl Environ Microbiol* 59:800–806
49. Keyser P, Pujar BG, Eaton RW and Ribbons DW (1976) Biodegradation of the phthalates and their esters by bacteria. *Environ Health Perspect* 18:159–166
50. Kimura T and Ito Y (2001) Two bacterial mixed culture systems suitable for degrading terephthalate in wastewater. *J Biosci Bioeng* 91:416–418
51. Kleerebezem R, Hulshoff Pol LW and Lettinga G (1999) The role of benzoate in anaerobic degradation of terephthalate. *Appl Environ Microbiol* 65:1161–1167
52. Kleerebezem R, Hulshoff Pol LW and Lettinga G (1999) Anaerobic degradation of phthalate isomers by methanogenic consortia. *Appl Environ Microbiol* 65:1152–1160
53. Kleerebezem R, Pol LW and Lettinga G (1999) Anaerobic biodegradability of phthalic acid isomers and related compounds. *Biodegradation* 10:63–73
54. Koch HM, Drexler H and Angerer J (2003) An estimation of the daily intake of di(2-ethylhexyl)phthalate (DEHP) and other phthalates in the general population. *Int J Hyg Environ Health* 206:77–83
55. Krauskopf LG (1973) Studies on the toxicity of phthalates via ingestion. *Environ Health Perspect* 3:61–72
56. Krishnan S, Prabhu Y and Phale P (2004) *o*-Phthalic acid, a dead-end product in one of the two pathways of phenanthrene degradation in *Pseudomonas* sp. strain PP2. *Indian J Biochem Biophys* 41:227–232
57. Latini G (2005) Monitoring phthalate exposure in humans. *Clin Chim Acta* 361:20–29
58. Li J and Gu JD (2006) Biodegradation of dimethyl terephthalate by *Pasteurella multocida* Sa follows an alternative biochemical pathway. *Ecotoxicology* 15:391–397

59. Li LH, Jester WF and Orth JM (1998) Effects of Relatively Low Levels of Mono-(2-Ethylhexyl) Phthalate on Cocultured Sertoli Cells and Gonocytes from Neonatal Rats. *Toxicology and Applied Pharmacology* 153:258–265
60. Lovekamp-Swan T and Davis BJ (2003) Mechanisms of phthalate ester toxicity in the female reproductive system. *Environ Health Perspect* 111:139–145
61. Lu KY, Tseng FW, Wu CJ and Liu PS (2004) Suppression by phthalates of the calcium signaling of human nicotinic acetylcholine receptors in human neuroblastoma SH-SY5Y cells. *Toxicology* 200:113–121
62. Ludwig ML, Ballou DP and Noodelman L (2001) Phthalate dioxygenase reductase. In: *Handbook of Metalloproteins*, Vol 1 (Messerschmidt A, Huber R, Poulos T and Weighardt K eds), John Wiley and Sons, Co., New York, pp 652–667
63. Malterud KE, Rydland KM and Haugli T (1999) Inhibition of 15-lipoxygenase by phthalate plasticizers *Bull Environ Contam Toxicol* 62:352–355
64. Mampel J, Providenti MA and Cook AM (2005) Protocatechuate 4,5-dioxygenase from *Comamonas testosteroni* T-2: biochemical and molecular properties of a new subgroup within class III of extradiol dioxygenases. *Arch Microbiol* 183:130–139
65. Marcel YL (1973) Determination of di-2-ethylhexyl phthalate levels in human blood plasma and cryoprecipitates. *Environ Health Perspect* 3:119–121
66. Maruyama K, Akita K, Naitou C, Yoshida M and Kitamura T (2005) Purification and characterization of an esterase hydrolyzing monoalkyl phthalates from *Micrococcus* sp. YGJ1 *J Biochem (Tokyo)* 137:27–32
67. Maruyama K, Shibayama T, Ichikawa A, Sakou Y, Yamada S and Sugisaki H (2004) Cloning and characterization of the genes encoding enzymes for the protocatechuate meta-degradation pathway of *Pseudomonas ochraceae* NGJ1. *Biosci Biotechnol Biochem* 68:1434–1441
68. Moody JD, Freeman JP, Doerge DR and Cerniglia CE (2001) Degradation of phenanthrene and anthracene by cell suspensions of *Mycobacterium* sp. strain PYR-1. *Appl Environ Microbiol* 67:1476–1483
69. Moore NP (2000) The oestrogenic potential of the phthalate esters. *Reprod Toxicol* 14:183–192
70. Nallii S, Cooper DG and Nicell JA (2002) Biodegradation of plasticizers by *Rhodococcus rhodochrous*. *Biodegradation* 13:343–352
71. Niazi JH, Prasad DT and Karegoudar TB (2001) Initial degradation of dimethylphthalate by esterases from *Bacillus* species. *FEMS Microbiol Lett* 196:201–205
72. Nishioka T, Iwata M, Imaoka T, Mutoh M, Egashira Y, Nishiyama T, Shin T and Fujii T (2006) A mono-2-ethylhexyl phthalate hydrolase from a *Gordonia* sp. that is able to dissimilate di-2-ethylhexyl phthalate. *Appl Environ Microbiol* 72:2394–2399
73. Nomura Y, Harashima S and Oshima Y (1989) A simple method for detection of enzyme activities involved in the initial step of phthalate degradation in microorganisms. *J Ferment Bioeng* 67:291–296
74. Nozawa T and Maruyama Y (1988) Anaerobic metabolism of phthalate and other aromatic compounds by a denitrifying bacterium. *J Bacteriol* 170:5778–5784
75. Orville AM, Lipscomb JD and Ohlendorf DH (1997) Crystal structures of substrate and substrate analog complexes of protocatechuate 3,4-dioxygenase: endogenous Fe³⁺ ligand displacement in response to substrate binding. *Biochemistry* 36:10052–10066
76. Overhage J, Kresse AU, Priefert H, Sommer H, Krammer G, Rabenhorst J and Steinbuechel A (1999) Molecular characterization of the genes *pcaG* and *pcaH*, encoding protocatechuate 3,4-dioxygenase, which are essential for vanillin catabolism in *Pseudomonas* sp. strain HR199. *Appl Environ Microbiol* 65:951–960
77. Pan G, Hanaoka T, Yoshimura M, Zhang S, Wang P, Tsukino H, Inoue K, Nakazawa H, Tsugane S and Takahashi K (2006) Decreased serum free testosterone in workers exposed to high levels of di-*n*-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP): a cross-sectional study in China. *Environ Health Perspect* 114:1643–1648
78. Patil NK, Kundapur R, Shouche YS and Karegoudar TB (2006) Degradation of plasticizer di-*n*-butylphthalate by *Delftia* sp. TBKNP-05. *Curr Microbiol* 52:369–374
79. Prabhu Y and Phale PS (2003) Biodegradation of phenanthrene by *Pseudomonas* sp. strain PP2: novel metabolic pathway, role of biosurfactant and cell surface hydrophobicity in hydrocarbon assimilation. *Appl Microbiol Biotechnol* 61:342–351
80. Pujar BG and Ribbons DW (1985) Phthalate metabolism in *Pseudomonas fluorescens* PHK: purification and properties of 4,5-dihydroxyphthalate decarboxylase. *Appl Environ Microbiol* 49:374–376
81. Qiu YL, Sekiguchi Y, Hanada S, Imachi H, Tseng IC, Cheng SS, Ohashi A, Harada H and Kamagata Y (2006) *Pelotomaculum terephthalicum* sp. nov. and *Pelotomaculum isophthalicum* sp. nov.: two anaerobic bacteria that degrade phthalate isomers in syntrophic association with hydrogenotrophic methanogens. *Arch Microbiol* 185:172–182
82. Qiu YL, Sekiguchi Y, Imachi H, Kamagata Y, Tseng IC, Cheng SS, Ohashi A and Harada H (2004) Identification and isolation of anaerobic, syntrophic phthalate isomer-degrading microbes from methanogenic sludges treating wastewater from terephthalate manufacturing. *Appl Environ Microbiol* 70:1617–1626
83. Quan CS, Liu Q, Tian WJ, Kikuchi J and Fan SD (2005) Biodegradation of an endocrine-disrupting chemical, di-2-ethylhexyl phthalate, by *Bacillus subtilis* No. 66. *Appl Microbiol Biotechnol* 66:702–710
84. Rani M, Prakash D, Sobti RC and Jain RK (1996) Plasmid-mediated degradation of *o*-phthalate and salicylate by a *Moraxella* sp. *Biochem Biophys Res Commun* 220:377–381
85. Rogers KS, Boots MR and Boots SG (1972) Molecular interactions of six aromatic competitive inhibitors with bovine liver glutamate dehydrogenase. *Biochim Biophys Acta* 258:343–350
86. Rubin RJ and Jaeger RJ (1973) Some pharmacologic and toxicologic effects of di-2-ethylhexyl phthalate (DEHP) and other plasticizers. *Environ Health Perspect* 3:53–59
87. Sasoh M, Masai E, Ishibashi S, Hara H, Kamimura N, Miyauchi K and Fukuda M (2006) Characterization of the terephthalate degradation genes of *Comamonas* sp. strain E6. *Appl Environ Microbiol* 72:1825–1832

88. Schläfli HR, Weiss MA, Leisinger T and Cook AM (1994) Terephthalate 1,2-dioxygenase system from *Comamonas testosteroni* T-2: purification and some properties of the oxygenase component. *J Bacteriol* 176:6644–6652
89. Shelton DR, Boyd SA and Tiedje JM (1984) Anaerobic biodegradation of phthalic acid esters in sludge. *Environ Sci Technol* 18:93–97
90. Shigematsu T, Yumihara K, Ueda Y, Morimura S and Kida K (2003) Purification and gene cloning of the oxygenase component of the terephthalate 1,2-dioxygenase system from *Delftia tsuruhatensis* strain T7 FEMS. *Microbiol Lett* 220:255–260
91. Stingley RL, Brezna B, Khan AA and Cerniglia CE (2004) Novel organization of genes in a phthalate degradation operon of *Mycobacterium vanbaalenii* PYR-1. *Microbiology* 150:3749–3761
92. Suemori A, Kurane R and Tomizuka N (1993) Purification and properties of phthalate oxygenase from *Rhodococcus erythropolis* S-1. *Biosci Biotechnol Biochem* 57:1482–1486
93. Suemori A, Nikajama N, Kurane R and Nakamura Y (1995) Production of 3,4-dihydroxyphthalate from phthalate by a membrane-bound two-enzyme system from *Rhodococcus erythropolis*. *Appl Microbiol Biotechnol* 43:470–472
94. Sugatt RH, O'grady DP, Banerjee S, Howard PH and Gledhill WE (1984) Shake Flask Biodegradation of 14 Commercial Phthalate Esters. *Appl Environ Microbiol* 47: 601–606
95. Sugimoto K, Senda T, Aoshima H, Masai E, Fukuda M and Mitsui Y (1999) Crystal structure of an aromatic ring opening dioxygenase LigAB, a protocatechuate 4,5-dioxygenase, under aerobic conditions. *Structure* 7:953–965
96. Tarasev M, Kaddis CS, Yin S, Loo JA, Burgner J and Ballou DP (2007) Similar enzymes, different structures: Phthalate dioxygenase is an $\alpha(3)\alpha(3)$ stacked hexamer, not an $\alpha(3)\beta(3)$ trimer like “normal” Rieske oxygenases. *Arch Biochem Biophys*. 466:31–39
97. Teil MJ, Blanchard M and Chevreuil M (2006) Atmospheric fate of phthalate esters in an urban area (Paris-France). *Sci Total Environ* 354:212–223
98. Tepper LB (1973) Phthalic acid esters – an overview. *Environ Health Perspect* 3:179–182
99. Vamsee-Krishna C, Mohan Y and Phale PS (2006) Biodegradation of phthalate isomers by *Pseudomonas aeruginosa* PP4, *Pseudomonas* sp. PPD and *Acinetobacter lwoffii* ISP4. *Appl Microbiol Biotechnol* 72:1263–1269
100. Vega D and Bastide J (2003) Dimethylphthalate hydrolysis by specific microbial esterase. *Chemosphere* 51:663–668
101. Vetting MW, D'Argenio DA, Ornston LN and Ohlendorf DH (2000) Structure of *Acinetobacter* strain ADP1 protocatechuate 3,4-dioxygenase at 2.2 Å resolution: implications for the mechanism of an intradiol dioxygenase. *Biochemistry* 39:7943–7955
102. Wang Y, Fan Y and Gu JD (2003) Microbial degradation of the endocrine-disrupting chemicals phthalic acid and dimethyl phthalate ester under aerobic conditions. *Bull Environ Contam Toxicol* 71:810–818
103. Wang YP and Gu JD (2006) Degradability of dimethyl terephthalate by *Variovorax paradoxus* T4 and *Sphingomonas yanoikuyae* DOS01 isolated from deep-ocean sediments. *Ecotoxicology* 15:549–557
104. Wang YZ, Zhou Y and Zylstra GJ (1995) Molecular analysis of isophthalate and terephthalate degradation by *Comamonas testosteroni* YZW-D. *Environ Health Perspect* 103:9–12
105. Whittaker JW, Orville AM and Lipscomb JD (1990) Protocatechuate 3,4-dioxygenase from *Brevibacterium fuscum*. *Methods Enzymol* 188:82–88
106. Wilkinson CF and Lamb JC (1999) The potential health effects of phthalate esters in children's toys: a review and risk assessment. *Regul Toxicol Pharmacol* 30:140–155
107. Yan H and Pan G (2004) Increase in biodegradation of dimethyl phthalate by *Clostridium lumula* using inorganic carbon. *Chemosphere* 55:1281–1285
108. Yoshida R, Hori K, Fujiwara M, Saeki Y and Kagamiyama H (1976) Nonidentical subunits of protocatechuate 3,4-dioxygenase. *Biochemistry* 15:4048–4053
109. Yun SH, Yun CY and Kim SI (2004) Characterization of protocatechuate 4,5-dioxygenase induced from *p*-hydroxybenzoate-cultured *Pseudomonas* sp. K82. *J Microbiol* 42: 152–155
110. Zhu J, Phillips SP, Feng YL and Yang X (2006) Phthalate esters in human milk: concentration variations over a 6-month postpartum time. *Environ Sci Technol* 40: 5276–5281
111. Zylstra GJ, Olsen RH and Ballou DP (1989) Cloning, expression, and regulation of the *Pseudomonas cepacia* protocatechuate 3,4-dioxygenase genes. *J Bacteriol* 171:5907–5914
112. Zylstra GJ, Olsen RH and Ballou DP (1989) Genetic organization and sequence of the *Pseudomonas cepacia* genes for the alpha and beta subunits of protocatechuate 3,4-dioxygenase. *J Bacteriol* 171:5915–5921