Biochemical pathway and degradation of phthalate ester isomers by bacteria

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Abstract Degradation of dimethyl isophthalate (DMI) and dimethyl phthalate ester (DMPE) was investigated using microorganisms isolated from mangrove sediment of Hong Kong Mai Po Nature Reserve. One enrichment culture was capable of utilizing DMI as the sole source of carbon and energy, but none of the bacteria in the enrichment culture was capable of degrading DMI alone. In co-culture of two bacteria, degradation was observed proceeding through monomethyl isophthalate (MMI) ester and isophthalic acid (IPA) before the aromatic ring opening. Klebsiella oxytoca Sc and Methylobacterium mesophilicum Sr degraded DMI through the biochemical cooperation. The initial hydrolytic reaction of the ester bond was by K. oxytoca Sc and the next step of transformation was by M. mesophilicum Sr, and IPA was degraded by both of them. In another investigation, a novel bacterium, strain MPsc, was isolated for degradation of dimethyl phthalate ester (DMPE) also from the mangrove sediment. On the basis of phenotypic, biochemical and 16S rDNA gene sequence analyses, the strain MPsc should be considered as a new bacterium at the genus level (8% differences). This strain, together with a Rhodococcus zopfii isolated from the same mangrove sediment, was able to degrade DMPE aerobically. The consortium consisting of the two species degraded 450 mg/l DMPE within 3 days as the sole source of carbon and energy, but none of the individual species alone was able to transform DMPE. Furthermore, the biochemical degradation pathway proceeded through monomethyl phthalate (MMP), phthalic acid (PA) and then protocatechuate before aromatic ring cleavage. Our results suggest that degradation of complex organic compounds including DMI and DMPE may be carried out by several members of microorganisms working together in the natural environments.

Keywords Biochemical cooperation; degradation; phthalate ester; plasticizer; wetland

Introduction Dimethyl isophthalate (DMI) is widely used in the production of sodium dimethyl isophthalate-5-sulfonate (SIPM) for enhancing the chromaticity of polyethylene terephthalate (PET). The major sources releasing large amounts of DMI into the environment are from wastewater generated during production of dimethyl terephthalate (DMT) (Duffel, 1993), which contain high concentrations of DMI at 35–40% as a major by-product of DMT manufacturing processes (Fajardo et al., 1997). Microorganisms are responsible for the degradation of toxic organic chemicals in the environment and recycling of carbon (Alexander, 1981). Owing to the limited awareness of ecological significance compared with other endocrine-disrupting phthalate esters (Kleerebezem et al., 1999), biodegradation of DMI has not been thoroughly studied.

Phthalates and phthalate esters are not only widely used as additives in plastics manufacturing and moulding to improve mechanical properties of the plastic resin, but also in paints, adhesives, cardboard, lubricants and fragrances (Giam et al., 1978; Nilsson 1994). Phthalates have been detected widely in environment (Giam et al., 1984), including
ground, river, drinking water, lake and marine sediments (Sullivan et al., 1982), landfills (Jonsson et al., 2003). Six phthalate esters including dimethylphthalate (DMPE), diethylphthalate, di-n-propylphthalate, di-n-butylphthalate, di-2-ethylhexylphthalate, and di-n-octylphthalate are designated as priority pollutants by the United States Environmental Protection Agency (US EPA 1992). As endocrine-disrupting chemicals, some phthalates may also interfere with the reproductive system and normal development of animals and humans (Allsopp et al., 1997; Gray et al., 1999; Jobling et al., 1995).

Several microorganisms showing ability to degrade isomers of phthalate ester isomers have been isolated from the activated sludge associated with a chemical manufacture (Aleshchenkova et al., 1997). A DMI-hydrolyzing enzyme purified from Rhodococcus erythropolis hydrolyzed DMI and dimethyl phthalate (DMP) readily while the other isomer dimethyl terephthalate (DMT) was hardly hydrolyzed by the same enzyme. This study showed that the initial hydrolysis reaction of this class of chemicals was carboxylic position specific (Gu et al., 2004). However, the complete mineralization of DMI appears to be more pronounced with mixed populations of selective microorganisms (Gu et al., 2004). Microbial degradation is the principal route for completely mineralization of phthalate in natural environments (Staples et al., 1997). Although considerable research has been conducted on the biodegradation of phthalate and DMPE over the last decade (Chang and Zylstra 1998; Kleerebezem et al., 1999; Roslev et al., 1998; Sugatt et al., 1984; Wang et al., 1996), few studies have examined the microorganisms involved in the degradation at pure culture level. Microorganisms from activated sludge were investigated for their role in the degradation of DMPE and phthalate, and no single species of bacteria from culture was capable of degrading DMPE (Wang et al., 2003b,c).

Mai Po Marshes (22° 30'N, 114° 02'E), situated at the northwestern corner of Hong Kong Special Administration Region, China, has been a Wetland of International Importance under the Ramsar Convention since 1995 in an effort to protect the wide range of natural and man-made habitats in the area, and indigenous and migratory birds (Tsim and Lock 2002). This area has one of the largest and most diverse mangrove communities along the south China coast and a large expanse of intertidal mudflat (Lau and Chu 1999), which is a feeding ground for the birds. Located closely to the densely populated cities, the Mai Po Marshes is suffering from increasing environmental stresses due to the pollutant from rapid urbanization and industrialization of the region. In particular, the Shenzhen River is one of the major sources of pollution. This may also suggest that mangrove sediments might harbor different groups of pollutant-degrading microorganisms due to the constant discharge from the river.

In this paper, we report that biochemical cooperation between microorganisms from mangrove sediment is involved in metabolism of DMI and DMPE, and a novel bacterium was involved in the consortium capable of degrading DMPE.

Materials and methods

Enrichment culture

The initial bacterial culture was established by adding 1.0 g of mangrove sediment taken from Mai Po Nature Reserve of Hong Kong into 100 ml of a mineral salt medium (MSM) in a 250 ml Erlenmeyer flask with DMI (starting concentration 100 mg/l) or DMPE (250 mg/l) as the sole source of carbon and energy. Similar approach was reported elsewhere (Gu et al., 2004; Wang et al., 2003a–c). The MSM consisted of the following chemicals (mg/l): (NH₄)₂SO₄ 1,000, KH₂PO₄ 800, K₂HPO₄ 200, MgSO₄·7H₂O 500, FeSO₄·10, CaCl₂·50, NiSO₄·32, Na₂O₇·H₂O 7.2, (NH₄)₆Mo₇O₂₄·H₂O 14.4, ZnCl₂·23, CoCl₂·6H₂O 21, CuCl₂·2H₂O 10 and MnCl₂·4H₂O 30, and the initial pH of the culture medium was adjusted with HCl or NaOH to 7.0 ± 0.1.
Identification of bacteria

The primary identification of bacteria was carried out using the API 20NE Multitest Kit (BioMerieux, Marcy l’Etoile, France) after Gram staining of the isolates after purification and streaking on agar plates. Tests were carried out following the instructions as described in the manufacture’s manual (bioMerieux 07615C, 1998). Each isolated bacterium and different combinations were then used and tested for their degradation ability on DMI or DMPE. One consortium of two pure species (strain MPpc and MPsc) showed the ability to degrade DMPE.

Chemical analysis

In preparation for HPLC analysis, thawed culture samples from DMI or DMPE degradation experiments were centrifuged (12,000 \(\times\) g) and filtered through PVDF or Nylon Acrodisc Minispike syringe filters (0.2 \(\mu\)m pore size) (Pall Gelman Laboratory, Ann Arbor, Michigan). Chemical concentration in the culture sample was determined by using High-performance Liquid Chromatograph (HPLC) (Agilent 1100 series, Agilent Technologies, California) equipped with a diode array UV-visible detector set at 254 nm (primary wavelength), 236 nm (secondary wavelength), and 280 nm as background reference. Samples (20 \(\mu\)l) were injected onto a Hypersil ODS C8 column (4.6 mm \(\times\) 250 mm). The mobile phase consisted of (A) \(\text{H}_2\text{O}\) containing 0.12 mol/L ammonium acetate and (B) methanol. Gradients were as follows: (1) 0–6 min \(B\) was held at 20%, (2) 6–10 min \(B\) increased from 20% to 60%, (3) \(B\) was held at 60% for 10 min, (4) \(B\) decreased to 20% in 6 min. DMPE, MMPE, PA, DMI, MMI and IPA were quantified using external standards after calibration.

Biomass measurement

During degradation, the microbial biomass was determined by optical density measurements at 600 nm spectrophotometrically using an UV 1201 (Shimadzu Co., Kyoto, Japan).

16S rDNA

One milliliter overnight culture of the bacterium grown in NB was centrifuged and the cell pellet was suspended in 200 \(\mu\)l extraction buffer (100 mM Tris, 100 mM EDTA, 200 mM NaCl, 1% Polyvinylpyrrolidone (w/v), 2% CTAB pH 8.0). Then 200 \(\mu\)l SDS buffer consisting of 2% SDS (w/v), 10 mM Tris, and 200 mM NaCl (pH 8.0) was added. The suspension was extracted with 400 \(\mu\)l phenol/Tris–HCl, 400 \(\mu\)l phenol and chloroform–isoamyl alcohol (25:24:1) and 400 \(\mu\)l chloroform/isoamyl alcohol (24:1) by centrifugation at 16000 rpm for 5 min, respectively. Bacterial DNA was precipitated in 400 \(\mu\)l isopropanol at \(-20^\circ\)C for 1 h and centrifuged at 14000 rpm for 20 min. DNA was vacuum-dried, dissolved in 50 \(\mu\)l sterile distilled water and stored at \(-20^\circ\)C before further experiment. Concentration of DNA was determined using Eppendorf BioPhotometer.

The PCR mixture (50 \(\mu\)l) contained the bacterial DNA, 0.5 \(\mu\)M primers (for the first 527-bp fragment), PCR buffer (10 mM Tris–HCl at pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.01% gelatin), a 200 \(\mu\)M concentration of each dNTP, and 1.0 U of Taq polymerase.

Multiple alignment of sequences was carried out using BioEdit, version 5.0.9 (Depart...
ment of Microbiology, North Carolina State University, U.S.). The phylogenetic analysis was conducted by the neighbor-joining method using the MEGA (Molecular Evolutionary Genetic Analysis) program package, version 2.1 (http://www.megasoftware.net/; Nei and Kumar, 2000).

**Scanning electron microscopy**

Culture of the bacteria used in this study was filtered on a 0.2 μm pore size polycarbonate membrane filter (Osmonics, Livemore, California). The membrane filters were prepared for SEM examination following the procedures of fixing initially in 2% glutaraldehyde and then 1% OsO₄, washing with cacodylate buffer, dehydration in a series of increasing ethanol concentrations, critical-point drying in liquid CO₂, and coating with gold–palladium as described elsewhere (Gu et al., 1996). The prepared samples were then observed under a Leica Cambridge S440 Scanning electron microscope.

**Results and discussion**

**Degradation of DMI**

Degradation of DMI was repetitively observed in the enrichment culture when DMI was the sole source of carbon and energy. Subsequently, bacterial isolates were obtained from the enrichment culture for further investigation with pure cultures. Each of the three bacteria isolated from DMI enrichment culture was tested for their abilities to grow on minimal agar plates and in liquid culture media supplemented with DMI, monomethyl isophthalate (MMI) or isophthalate (IPA) as the sole source of carbon and energy. All three isolates were capable of utilizing IPA as sole sources of carbon and energy both on agar plates and in liquid cultures. However, strain Sc and Sr metabolized DMI and MMI, respectively, only in liquid cultures. Microscopic examination revealed that both strains Sc and Sr were Gram-negative, rod shape, but strain Sw was Gram-positive and rod shape. Strain Sc was identified as *Klebsiella oxytoca* with 96.1% similarity by API 20E while strain Sr was *Methylobacterium mesophilicum* with 93.7% similarity by API 20NE system.

Degradation of DMI was investigated using *K. oxytoca* Sc or *M. mesophilicum* Sr. DMI could not be degraded by either of the two isolates alone to the completion of mineralization. Specifically, *K. oxytoca* Sc was able to transform DMI to MMI and the intermediate accumulated in the culture medium without further decrease over extended period of incubation (Figure 1). However, *M. mesophilicum* Sr was able to degrade the intermediate MMI quickly to IPA. Furthermore, both species were shown to metabolize IPA.

It appears that completed degradation of the phthalate esters require biochemical cooperation between selective species of bacteria from community of the natural environment. Two major intermediates of biochemical transformation of DMI were identified as

![Figure 1](https://iwaponline.com/wst/article-pdf/52/8/241/434297/241.pdf)
MMI and IPA, respectively, by a combination of methods as described elsewhere (Wang et al., 2003a,b). Analysis of the culture aliquot on HPLC revealed that DMI was completely hydrolyzed with subsequent formation of MMI and IPA sequentially, which can be mineralized to CO₂ and H₂O when both species were present at the same time.

Transformation of DMI (figure 2) requires the participation of _K. oxytoca_ Sc first forming monomethyl isophthalate and then _M. mesophilicum_ Sr forming isophthalate acid (IPA). Interestingly, both species were equally capable of utilizing IPA. Similar biochemical process was also confirmed early using an enrichment culture obtained from activated sludge (Niazi et al., 2001; Wang et al., 2003a). When pure cultures of bacteria were isolated, they were not capable of degrading the phthalate ester by individual species, a combination of them was observed to completely degrade the substrate (Wang et al., 2003b). Phthalate acid is degradable by single species and _Comamonas acidovorans_ fy-1 mineralized this substrate at concentration as high as 20,000 mg/l (Fan et al., 2004).

A consortium of at least two microorganisms was shown to be effective in degradation of the two chemicals. Biochemical cooperation in degradation may be more widely present in the natural environment. DMI was rapidly metabolized in culture of _K. oxytoca_ Sc with DMI as the sole sources of carbon and energy and completely transformation of DMI was achieved within 36 h at an initial concentration of 108 mg l⁻¹. One transformation intermediate was observed and accumulated without further transformation even after 40 d of incubation (Figure 1). It should be pointed out here that both hydrolysis for the initial removal of ester bonds involved identical chemical structure, but the sequential arrangement of the two bacteria in the hydrolysis imply that biochemically speaking, the ester hydrolyzing enzymes are different structurally. Further investigation to the molecular and proteomics basis will reveal the fundamental differences between the ester hydrolytic enzymes involved.

Degradation of DMPE

Four bacteria were isolated from the enrichment culture using DMPE as the sole carbon and energy sources, but none of them could mineralize DMPE alone. The consortium used in this investigation was reconstituted from two bacterial isolates (strain MPpc and MPsc). Strain MPsc was Gram-negative, non-mobile, oxidase positive. Scanning electron microscopy revealed that the cell of strain MPsc was short-rod and slightly pointed at both ends (Figure 3). Based on the 16S rDNA sequence, strain MPpc was determined as _Rodococcus zopfii_ with difference less than 0.00%, and named _Rodoccocus zopfii_ strain MPpc. Similarly strain MPsc was identified as a new bacterium on the genus level with 16S rDNA difference of 8.00%. The 16S rDNA sequence for strain MPsc was compared with that of _E. coli_ and 18 strains deposited in GeneBank that are similar to MPsc, followed by construction of a phylogenetic tree (Figure 3). Strain MPsc belongs to the group of β-proteobacteria, and its nearest phylogenetic neighbor is an unculturable bacterium clone.

The consortium of strain MPsc and _Rodococcus zopfii_ strain MPpc was capable of degrading 450 mg/l DMPE within 78 hours (data not shown). Meanwhile, the biomass of

![Figure 2 A proposed degradative biochemical pathway carried out by _Methyllobacterium mesophilicum_ and of dimethyl isophthalate by _Klebsiella oxytoca_ isolated from mangrove sediment](https://iwaponline.com/wst/article-pdf/52/8/241/434297/241.pdf)
the consortium increased as the DMPE depleted. In contrast, the concentration for DMPE in sterilized controls was constant during the whole experiments, which indicated that the degradation of DMPE due to natural hydrolysis and photolysis on was negligible. Three intermediates were identified as mono-methyl phthalate (MMP), phthalic acid (PA) and protocatechuate during DMPE degradation under aerobic condition. MMP appeared first after 12 h of incubation and accumulated to the peak level of 109 mg/l after 60 h, and PA showed similar trend as MMP at much lower concentration. Both of them were not detectable after 80 h. A small amount of protocatechuate was also produced before 70 h of inoculation, and was also not detectable after 90 h. From the intermediate information, the proposed metabolic pathway is constructed. Degradation of DMPE followed two steps of ester hydrolysis resulting in MMP and PA, and further decarboxylation resulting in protocatechuate before the cleavage of aromatic ring.

A new bacterium involved in DMPE degradation, strain MPsc, was isolated from the sediment of Mai Po and it is relatively selective on carbon source. Among 18 carbon sources tested, only malate, acetate and phenyl-acetate could be utilized. The neighbor-joining phylogenetic tree showed that strain MPsc was a member of the β-subclass of the Proteobacteria and formed a tight distinct cluster together with an uncultured bacterium clone (Figure 3). Although this cluster grouped with a cluster of Comamonas spp., the bootstrap frequency value for this node was low (Figure 3). Based on the biochemical identification and phylogenetic analysis, it is more appropriate to assign strain MPsc into a new genus than a previously established one. Three closely related genera are Comamonas, Acidovorax and Delftia, suggesting that the new genus should belong to the family Comamonadaceae.

No report is available on description of DMPE-degrading bacteria from mangrove sediment. Current information on DMPE-degrading bacteria was mostly restricted to wastewater and activated sludge (Wang et al., 1996, 2003c) or mixed culture enriched from soil (Vega and Bastide 2003). Although consortium of bacteria from the activated sludge was capable of utilizing DMPE as the sole carbon and energy source, more than two bacterial species were required for the degradation (Wang et al., 2003c). The observed phenomenon was due to the fact that DMPE degradation requires the cooperation among various enzymes that belongs to different bacterial species. It is apparent that hydrolysis of ester bond is the initial step in the degradation of DMPE producing sequentially MMP, PA and protocatechuate before the cleavage of the aromatic ring.
ring. The metabolic pathway proposed in this study is similar to previous study using activated-sludge bacteria (Wang et al., 1997) or mixed soil bacteria (Vega and Bastide, 2003).

In conclusion, degradation of DMI and DMPE can be achieved with mixed culture of bacteria from mangrove sediment and complete degradation requires the biochemical cooperation between selective species of bacteria. Initial reaction involves hydrolysis of the ester bonds of both chemicals. Strain MPsc, isolated from mangrove sediment, was involved in aerobic DMPE degradation and is a new bacterium to the genus level based on 16S rDNA sequencing. The reconstituted consortium consisting strain MPsc and Rodococcus zopfii MPpc showed the ability in degrading DMPE through MMP, PA and protocatechuate as detectable intermediates. Our results collectively suggest that degradation of complex organic compounds including DMPE may be carried out by several different microorganisms working together in the natural environments.

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