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Degradation of the endocrine-disrupting dimethyl phthalate carboxylic ester by *Sphingomonas yanoikuyae* DOS01 isolated from the South China Sea and the biochemical pathway

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ABSTRACT

Bacteria capable of using dimethyl phthalate (DMP) as the sole carbon and energy source were isolated from the sediments collected at a depth of 1340 m from the South China Sea. *Sphingomonas yanoikuyae* DOS01, identified based on 16S rRNA gene sequence, utilized DMP from an initial level of 180 mg l^{-1} to non-detectable in 35 h at 30 °C, the optical density (OD_{600}) values increased over the time of incubation. Degradation intermediate monomethyl phthalate (MMP) accumulated up to 21.3 mg l^{-1} and then disappeared in the culture medium. When MMP or another intermediate phthalate (PA) was used as the sole substrate, this strain was only capable of degrading MMP, but not PA. Total organic carbon (TOC) analysis of the culture medium suggested that both DMP and MMP were mineralized, but not PA. This strain from the deep-ocean sediment transforms DMP to MMP using a common biochemical pathway for DMP as reported before. Further esterase activity assays indicated that the enzyme induced by MMP has higher affinity than that by DMP for the substrate *p*-nitrophenyl acetate. Our results indicated that complete degradation of DMP by this marine microorganism may involve a new biochemical pathway.
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1. Introduction

Phthalate acid esters (PAEs) are a large class of chemicals widely used in the plastics manufacturing industries as plasticizers. They have been used in the production of PVC for over half a century and in other industrial products relying on softness and flexibility such as home furniture, daily used containers and children's toys (Wilkinson and Lamb IV, 1999; Gu et al., 2000; Gu, 2003a,b, 2007). In Germany alone, consumption of di(2-ethylhexyl) phthalate (DEHP) was over 250,000 tons between 1994 and 1995, and reached 400,000 tons more recently (Fromme et al., 2002). As they have been detected in samples from a wide range of environments, concerns over PAEs responsible for human endocrine-disrupting have been speculated and investigated. In the landfill, a high concentration of PAEs in the leachate was detected including some of their key degradation intermediates (Baikova et al., 1999; Fromme et al., 2002; Schwarzbauer et al., 2002; Pinyakong et al., 2003).

Studies have been conducted for a detailed examination of PAEs' acute toxicity (Doull et al., 1999; Call et al., 2001; Schwarzbauer et al., 2002). Dimethyl phthalate (DMP) was also assayed for its toxicity, but no carcinogenic activity in male and female rats has been demonstrated in the relevant investigations (National Toxicology Program, 1995). However, uncertainty of its effects on the male and female mice tested has not been resolved in terms of carcinogenic activity. Large uptake of DMP instigated central nervous depression and noticeable kidney damage (Autian, 1973). Other bioassays using *Hyalella azteca*, *Chironomus tentans*, and *Lumbriculus variegates*, showed that *Hyalella azteca* was most sensitive to DMP with mechanism of narcosis response and a mortality rate of 95% was observed at 50 mg l^{-1} of DMP (Call et al., 2001).

Microorganisms have been found for elimination of DMP in various environments. Both *Pseudomonas fluorescens* and *Pseudomonas testosteroni* were reported with ability to metabolize DMP as a sole carbon and energy source, with distinct benzene ring cleavage pathway (Keyser et al., 1976; Gu, 2003a,b, 2007). Eaton and Ribbons (1982) described that *Micrococcus* sp. strain 12B could mineralize DMP with a main pathway and a minor one. Another Gram positive bacterium *Bacillus* sp. also was found to degrade DMP as a sole carbon and energy source (Niazi et al., 2001). In addition, microorganisms from activated sludge (Wang et al., 2003a–c, 2004), mangrove (Fan et al., 2004; Gu et al., 2005; Li and Gu, 2006a,b, 2007; Li et al., 2005a,b; Xu et al., 2005a,b, 2007a,b) and deep-ocean (Wang and Gu, 2006a,b; Wang et al., 2008) have been isolated for

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degradation of this class of chemical and consortia of microorganisms were also found to be involved in the mineralization (Vega and Bastide, 2003; Li and Gu, 2006b, 2007). During the degradation, the intermediates of monomethyl phthalate (MMP), phthalate (PA) and protocatechuate were normally yielded by the strain, followed by cleavage process (Eaton and Ribbons, 1982; Eaton, 2001; Vega and Bastide, 2003).

Since there is very limited information available on DMP degrading bacteria from deep-ocean environment and their degradation pathway, it is valuable to pursuit an investigation on isolation and application of bacteria found in high salt environment. The objectives of this study were to isolate microorganisms capable of degrading DMP from sediments sampled from South China Sea, characterize these microorganisms, establish possible pathway of degradation processes, and explore the enzyme active of such microorganisms.

2. Materials and methods

2.1. Enrichment and isolation of bacteria

The bacteria used in the degradation experiment were isolated from the sediments collected from a depth of 1340 m on sea floor of South Chinese Sea, which was located at 109°25.079′, 9°17.038′ (longitude, latitude) by a scientific survey ship of the South China Sea Institute of Oceanography, Chinese Academy of Sciences in Guangzhou, P.R. China. The samples were preserved at −20 °C immediately after being collected before use.

In the initial enrichment cultures, about 5 g (wet weight) of sediments were introduced into 250 ml Erlenmeyer flasks of 100 ml sterile culture medium containing a mineral salt medium (MSM) as following (mg/l): $(NH_4)_2SO_4$ 1000, KH_2PO_4 200, K_2HPO_4 800, $MgSO_4 \cdot 7H_2O$ 500, $FeSO_4$ 10, $CaCl_2$ 50, $NiSO_4$ 32, $Na_2BO_7 \cdot H_2O$ 7.2, $(NH_4)_6Mo_7O24 \cdot H_2O$ 14.4, $ZnCl_2$ 23, $CoCl_2 \cdot H_2O$ 21, $CuCl_2 \cdot 2H_2O$ 10, and $MnCl_2 \cdot 4H_2O$ 30; and 200 mg l^{-1} DMP (Wang et al., 2003a). The sterile stock of DMP solution filtered through 0.2 μm-pore-size membrane filter (Osmonics, Livemore, CA) was mixed into the MSM which was previously autoclaved at 120 °C for 20 min, adjusted to pH 7.0 ± 0.1. The flasks were incubated in a shaker (INNOVA 4340 incubation shaker, New Brunswick Scientific, NJ), at 150 rpm and 30 °C. After 1 week of incubation, about 2 ml liquid cultures were transferred into a freshly prepared culture medium to establish new enrichment transfer culture. Subjected to at least three times of such enrichment transfers, bacteria in the enrichment culture were isolated and purified.

2.2. Identification and characterization of the strains

The bacterial colonies on the NB agar plates were picked for Gram staining. When Gram negative, API 20 NE Multitest System (bioMerieux, Marcy l'Etoile, France) was applied to characterize the isolates biochemically as described elsewhere (Gu and Mitchell, 2001). The procedure of operation was also described in the instructions of the manufacturer.

The bacterial isolates were further characterized using 16S rRNA gene sequencing methods (Wang et al., 1996; Di Cello et al., 1997). One ml overnight culture of bacterium grown in Nutrient Broth incubated in an INNOVA 4340 Incubator Shaker (New Brunswick Scientific, NJ) at 150 rpm and 30 °C in the dark was centrifuged (5 min, 2000 × g). The cell pellet was washed four times with phosphate – buffer saline (PBS, 0.05 M, pH 7.4), followed by once with water de-ionized and re-suspended in 0.1 ml of distilled water, diluted in 0.1 ml of 1% Triton X-100, heated at 100 °C for 5 min in boiling water, and immediately cooled on ice. Cells were directly centrifuged at 9000 × g and then were washed twice with PBS and once with water, repeated the re-suspension and cell breaking process. 2 μl of DNA from the fractionated cells was mixed with 20 μl of Promega *Taq* buffer containing 1.5 mM $MgCl_2$ with 150 ng of each primers, 250 mM each dNTP, and 1.0 U of *Taq* DNA polymerase (Invitrogen Co., CA). The reaction mixtures were processed in a thermocycler at 95 °C for 1 min and 30 s and then cycled 35 times through the following temperature profile: 95 °C for 30 s, annealing temperature (T_a) for 30 s, and 72 °C for 4 min. The T_a was 60 °C for the first five cycles, 55 °C for the next five cycles, and 50 °C for the last 25 cycles. Finally, the mixtures were incubated at 72 °C for 10 min and then at 60 °C for 10 min. Nucleotide sequences were determined with the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, RRC-West, MBRB 3260). The sequence of the DNA with accession number AY878409 was aligned and compared with the data in the GenBank.

The morphology of the strains was observed using scanning electron microscopy (SEM). The bacterium was incubated in the Nutrient Broth medium (Difco Lab., Detroit, MI) overnight, and filtered onto the 0.2 μm microfilter (Osmonics, Livemore, CA). The bacteria on filter were fixed in 2% glutaraldehyde and 1% OsO_4, washed with cacodylate buffer. The bacteria were dehydrated in an increasing concentration series of ethanol from 50 to 100%, and subject to drying in liquid CO_2. Before observed under the SEM, the filter was also coated with gold-palladium as described elsewhere (Gu et al., 1996).

2.3. Degradation experiments

The bacterial isolate capable of degrading DMP was prepared in the diluted nutrient broth (NB) culture medium (containing 0.13% NB) over night on shaker before being exposed to substrates medium. After over night incubation, 1 ml culture aliquot was pipetted and transferred into a sterile Eppendorf tube and centrifuged at 7000 × *g* for 5 min (Minispin plus, Eppendorf, Hamburg, Germany), and the supernatant was discarded to remove the residual NB. The same volume of DMP medium was refilled into the tube and mixed thoroughly. The bacterial suspension was transferred into the flask containing 100 ml aseptically prepared DMP culture medium sterilized by passing through 0.2 μm syringe filter (Iwaki Glass, Japan) into autoclaved flask. Triplicate of such culture medium were incubated in the INNOVA shaker (New Brunswick Scientific, NJ) at 150 rpm and 30 °C. The control was set up with chemical but without inoculation of bacteria. The MMP and PA degradation experiment were built up by method above. The initial concentration of MMP and PA were made at 250 and 110 mg l^{-1}, respectively. After regular time intervals, 1.3 ml culture medium were taken for measuring the optical density (OD_{600}) on a UV 1201 Spectrophotometer (Shimadzu Co., Kyoto, Japan) and preserved at −20 °C for optical density measurement and HPLC analysis.

The samples frozen were thawed at room temperature, filtered by passing through 0.2 μm syringe filter (Iwaki Glass, Japan) before analysis. DMP and intermediates were quantified on the Agilent 1100 HPLC systems (Agilent Technologies, CA) which was equipped with a column (Zorbax Eclipse XDB-C8, Agilent Technologies, CA) as described previously by others of this laboratory (Wang et al., 2003a). The mobile phase for the HPLC was 50:40:10 (methanol:water:phosphate pH 3.0, v/v), under condition of 30 °C and a flow rate of 1.0 ml min^{-1}. The UV absorption spectra of DMP and its intermediates were measured at 280 nm wavelength. The quantification was accomplished by external standard (concentration range from 10 to 1000 mg l^{-1}, $r^2 = 0.9998$).

2.4. Total organic carbon assay (TOC)

For investigating the completely degradation ability of the bacterial strain, total organic carbon assay was used. Culture media of DMP, MMP and PA were prepared in flasks with concentration of

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