Complete Degradation of the Endocrine-Disrupting Chemical Dimethyl Phthalate ester by *Flavobacterium* sp.

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The complete microbial degradation of dimethyl phthalate ester (DMPE) is described. DMPE is thought to be an endocrine-disrupting chemical. A pure culture (strain No. A-9) from soil sample capable of utilizing DMPE as the sole source of carbon and energy was identified as *Flavobacterium* sp. Degradation patterns of DMPE were observed on the high-performance liquid chromatogram (HPLC) of the culture filtrates of this strain, and growth of bacteria was measured as protein by the Kennedy and Fewson method. The growth yield of this strain was about 5.9 g of protein per mole of carbon source of DMPE, and was similar to that in the case of glucose as a carbon source. Complete degradation of DMPE had been achieved (1000 mg/l) in less than 2 days using *Flavobacterium* sp. strain No. A-9. The transient intermediates of DMPE were not detectable on the HPLC of the culture filtrates of this strain. This strain also degraded phthalic acid (PA) but could not degrade phthalic anhydride.

Key words —— dimethyl phthalate ester, complete degradation, sole carbon source, growth yield, *Flavobacterium*

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INTRODUCTION

In recent years, phthalate esters have attracted increasing attention owing to their widespread use, ubiquity in the environment, and endocrine-disrupting activity.[1,2] Phthalate esters are synthetic compounds used as plasticizers to improve mechanical properties of the plastic resin, particularly flexibility.[3] However, in order to provide the required flexibility, the phthalate plasticizer is not bound covalently to the resin and thus, is able to migrate into the environment.[4] Plasticizers are also widely used in building materials, home furnishings, transportation, clothing and, to a limited extent, in food and medical products. Because of the global utilization of plasticized polymers in large quantities, phthalates and their esters have been detected in every environment in which they have been soughed.[5] Known as endocrine-disrupting chemicals, selective phthalates may also interfere with the reproductive system and normal development of animals and humans.[6–8] Recently, many studies on phthalate and esters mainly focused on biodegradability, metabolic pathway by pure culture of microorganisms and removal of phthalate in wastewater treatment systems.[2,9–18] However, few studies have focused on the processes and degradation ability of bacteria. In the previous paper,[19] we reported the complete degradation of phthalic acid (PA) by *Flavobacterium*. In this paper, the complete degradation of degradation of DMPE by a new isolate (strain No. A-9) from soil sample is described together with the taxonomic characteristics of the strain.

MATERIALS AND METHODS

Materials —— DMPE (purity, 99%) was purchased from Kanto Chemical Co., INC. (Tokyo, Japan) and used without further purification. All other chemicals were guaranteed to be of the best grade commercially available.

Apparatus, Analysis, and Measurement of DMPE —— Spectrophotometric analysis was done using a Hitachi (Tokyo, Japan) 124 spectrophotometer, equipped with flow-through cell of 1.0 cm path length. High-performance liquid chromatography (HPLC) was done using jacketed stainless steel analytical column (4.6×150 mm i.d.) packed with Cosmosil $5C_{18}$-MS-II (5 μm). The mobile phase was CH_3OH-H_2O (80 : 20, v/v, pH 7.0) containing

0.05 M heptanesulfonic acid. The concentration of DMPE was routinely measured by HPLC on an apparatus equipped with UV detector set at 276 nm. Quantitation of DMPE was achieved through the use of a calibration curve. A set of standards (100–1000 μg/ml) produced daily by serial dilution was used. In the above concentration range, a linear response was obtained (regression equation $y = 792.46x$, correlation coefficient = 0.9999). Samples from bacterial cultures were filtered (0.45 μm pore diameter) before chromatography.

Growth Media and Isolation of Microorganisms —— The growth medium used for carbon-limited growth was 10 mM potassium phosphate buffer, pH 7.3, and contained 0.25 mM $MgSO_4$, 2% ammonium chloride as the sole source of nitrogen, trace elements (0.1% $FeSO_4 \cdot 7H_2O$, 0.01% $CuSO_4 \cdot 5H_2O$, 0.01% $ZnSO_4 \cdot 7H_2O$, 0.01% $MnSO_4 \cdot 7H_2O$ and 0.01% $CaCl_2$; supplied at 2 ml/l) and less than 0.5 mM DMPE as the sole source of carbon. The buffered solution of $MgSO_4$ was autoclaved, and the sterile solution of trace elements, DMPE, and ammonium chloride were added aseptically. The term, buffered salts, refers to 10 mM potassium phosphate buffer, pH 7.3, containing 0.25 mM $MgSO_4$. Enrichment cultures were used to obtain isolates able to utilize DMPE as a sole and limiting source of carbon for growth. Inocula for enrichments were prepared from soils from Kumamoto prefecture. Enrichment cultures were nonsterile and contained DMPE at 1000 μg/ml as a carbon source, buffered salts, ammonium chloride and 20% of preculture. Cultures were carried out with 30 ml medium in 200-ml Erlenmeyer flasks under aerobic condition for 7d at 37°C on a rotary shaker (200 rpm). All enrichments were subcultured three times into homologous medium. Positive enrichments were streaked on nutrient agar plates and growth medium agar plates containing DMPE at 200 μg/ml. A representative of each colony type was picked from the agar plates. Stock cultures of each isolate were maintained in growth medium agar slants containing DMPE at 200 μg/ml.

Taxonomic Characteristics of Strain No. A-9 —— The morphology of strain No. A-9 was studied by phase-contrast microscopy, and the Gram reaction was examined by using the Gram stain. Roch Oxi-Ferm and Enterotubes were used in combination with the method of Stanier *et al.*[20]

Quantification of Growth and DMPE Utilization —— Bacterial growth yields with limiting carbon source were determined at 37°C with 50 ml cultures in 200-ml Erlenmeyer flasks on a rotary shaker (200 rpm). Media containing carbon source at five concentrations between 0 and 5 mM carbon were inoculated (4% v/v) with cell suspension of bacterial culture induced to grow in a limiting amount of DMPE. The nitrogen source was 2% (final concentration) ammonium chloride for microorganism. DMPE in cultures was quantified by HPLC. Growth of bacteria was measured routinely as protein (rather than turbidity or dry weight) to eliminate trivial complication arising from the accumulation of lipid or carbohydrate storage polymers, which include factors interfering with bacterial growth measurement. Bacteria were treated with a final concentration of 0.5 M trichloroacetic acid and collected by centrifugation at 12000 rpm at 0°C for 20 min. The supernatant fluid was discarded. The resultant precipitate was suspended in 0.66 M NaOH, and protein was assayed according to the method of Kennedy and Fewson.[21]

RESULTS AND DISCUSSION

Enrichment and Isolate of Microorganisms Utilizing DMPE

Cultures were enriched under aerobic condition. Three pure cultures of bacteria were obtained from the aerobic enrichment culture, and no fungi were isolated.

DMPE-Degrading Activity of Isolates

The growth curves of three isolates were examined using DMPE as a sole source of carbon. Strains No. A-8, A-9 and A-10 could utilize DMPE as a sole source of carbon or degrade it. Strain No. A-9 could completely degrade DMPE (1000 mg/l) in less than 2 days, but strain No. A-8 and A-10 could not completely degrade (50–60%) DMPE in 2 days. Complete degradation of DMPE has been achieved (1000 mg/l) in 4 days using strain No. A-8 and A-10 (Table 1). Strain No. A-9 degraded DMPE more rapidly than strains No. A-8 and A-10. Strain No. A-9 was examined using DMPE as the sole source of carbon (Fig. 1). Figure 1 shows that the concentration of DMPE decreases inversely with respect to the increase of the growth yield of the strain No. A-9. The cultures without bacteria showed no change in the concentration of DMPE and negligible growth in the medium. The pH of the pure culture (strain No. A-9) was nearly constant throughout the cultivation time. These results suggest that the decrease

Table 1. Degradation of PA, DMPE, DEPE and phthalic anhydride using Strain No. A-1, -8, -9 and A-10

Substrate	Bacterial strain used	Time (days)	Degradation (%)
PA	A-1	2	100
	A-8	4	100
	A-9	2	100
	A-10	4	100
DMPE	A-1	7	No degradation
	A-8	4	100
	A-9	2	100
	A-10	4	100
DEPE	A-1	7	No degradation
	A-8	7	No degradation
	A-9	7	No degradation
	A-10	7	No degradation
Phthalic anhydride	A-1	7	No degradation
	A-8	7	No degradation
	A-9	7	No degradation
	A-10	7	No degradation

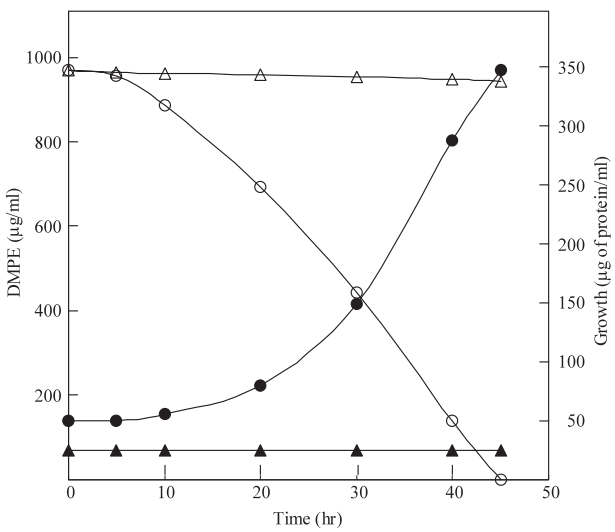

Fig. 1. Microbial Degradation of DMPE by Strain No. A-9

○, △, concentration of DMPE in medium; ●, ▲, growth measured as protein; ○, ●, with strain No. A-9, △, ▲, control (without any strain).

of DMPE is not due to precipitation by formation of salts or to decomposition by acid. The increase of growth yield was considered to be due to utilization of DMPE as a carbon source. As described above, strain No. A-9 was regarded as DMPE-degrading bacteria.

Taxonomic Characteristics of Strain No. A-9

Taxonomic characteristics of strain No. A-9 is summarized in Table 2. The strain No. A-9 was regarded as *Flavobacterium* sp. because it was Gram-negative, strictly aerobic, oxidase-positive, non-motile rods which grew aerobically with citrate as a carbon source. Urease reaction was strongly positive, whereas arginine dihydrolase and lysine decarboxylase reactions were negative. It grew with the production of a yellow pigment and without H_2S or indole production. In the previous paper,[19] we reported the complete degradation of PA by *Flavobacterium* strain No. A-1. The strain No. A-1 did not produce pigments in growth medium, but the strain No. A-9 produced pigments (yellow) in growth medium. The strain Nos. A-8 and A-10 were also regarded as *Flavobacterium* sp. and did not produce pigments in growth medium.

Table 2. Taxonomic Characterization of Strain No. A-9

Properties or tests	Strain No. A-9
Bacterial type	Rods
Mobility	Non-motile
Size	0.6 by 1.2 mm
Optimal temperature	37°C
Optimal pH	8.0
Gram stain	−
Oxidase	+
Catalase	+
Arginine dihydrolase	−
Lysine decarboxylase	−
Indole	−
Urease	+
Citrate	+
Acid production from carbon source	−
Ana-Glc[a)]	−
Aer-Glc[b)]	−
Xylose	−
Maltose	−
Mannitol	−
Sucrose	−

a) Glucose under anaerobic conditions, *b*) Glucose under aerobic conditions.

Quantification of DMPE Utilization for Growth

Preliminary evidence for the utilization of DMPE as the sole source of carbon was the growth yield of organisms (measured as protein) per mole of supplied carbon (Table 3). In strain No. A-9, the yield of cells per mole of DMPE was similar to the yield of this strain per mole of glucose (*e.g.*, 5.9 and 6.0 g of protein, respectively, for strain No. A-9). This implies that the DMPE carbon was quantitatively used by the bacteria for growth. In both cases, the measurable carbon-containing substrates were quantitatively removed from the growth medium (Table 3). The growth yields were the gradients of

Table 3. Growth Yields of Strain No. A-9 with DMPE as the Sole Source of Carbon

Substrate	Atom of C per mole of substrate	Bacterial strain used	C-source remaining after growth	Growth yield (g per mole of S)[a)]	Growth yield (g of protein per mole of C)[b)]
DMPE	10	A-9	None	59.1 ± 2.0	5.9 ± 0.2
Glucose	6	A-9	None	36.0 ± 0.9	6.0 ± 0.15

a) substrate, *b*) carbon: DMPE, glucose.

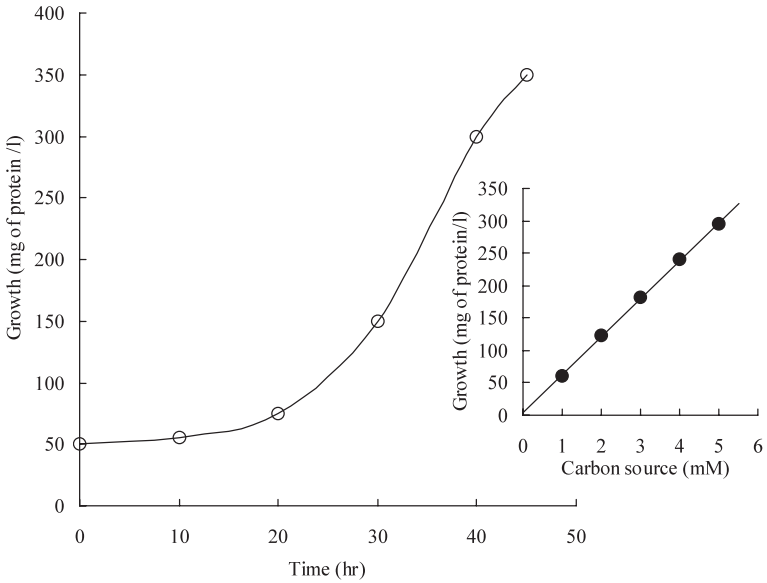

Fig. 2. Growth Curve of Strain No. A-9 with DMPE as the sole source of carbon
The insert is a plot of substrate concentration *vs.* the corresponding protein concentration.

lines obtained by plotting cell yield *vs.* intial concentration of carbon source. The cultures without combined carbon showed negligible growth. Additional controls were the following: DMPE was stable throughout the cultivation time and no carbon ion was taken up from the atmosphere. A typical growth curve of strain No. A-9 is shown in Fig. 2. The strain No. A-9 grew exponentially with DMPE as a sole source of carbon. The differential plot of substrate concentration *vs.* protein concentration (Fig. 2, insert) was linear, demonstrating that DMPE utilization was concomitant with growth. Similar data was obtained for strain No. A-9 growing with carbon ion as the sole and limiting carbon source (not shown). The growth yields in our experiment (Table 3) are consistent with the quantitative utilization of DMPE carbon as a carbon source of bacteria because the molar growth yields are similar to those in a review of bacterial cell composition.[22)] This indirect proof of quantitative DMPE utilization was supported by substrate disappearance (Table 3), which was usually concomitant with growth (Fig. 2). In summary, complete degradation of DMPE has been achieved (1000 mg/l) in less than 2 days using a single bacterial species, *Flavobacterium* sp. strain No. A-9, which was isolated from soil sample. Transient intermediates of DMPE degradation were not observed on the HPLC of the strain No. A-9 culture. This strain could also degrade PA, but could not degrade diethyl phthalate ester (DEPE) and phthalic anhydride (Table 1). Dioxygenases have been known for their ability to cleave the aromatic ring[3)] and it is likely that the site of cleavage is between the two carboxylmethyl groups, but not the carboxylethyl groups. It is possible that the site of cleavage is between the two carboxyl groups and two carboxylmethyl groups.

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