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Initial degradation of dimethylphthalate by esterases from *Bacillus* species

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Abstract

Dimethylphthalate (DMP), one of the phthalate esters, is used in the manufacture of plasticizers, insect repellents, and synthetic fibers, and contributes to environmental pollution. In the present study, we report a novel bacterium belonging to the *Bacillus* sp., which has the ability to utilize DMP as the sole source of carbon. The esterases from the cell-free extract of the *Bacillus* de-esterified DMP. Native polyacrylamide gel electrophoresis showed the presence of four isoesterases designated Et1–4. The isoesterases Et-4 and Et-1 showed a higher preference towards DMP hydrolysis as compared with Et-2 and 3. A megaplasmid of about 60 kb was detected in this bacterium. The ability of this bacterium to utilize DMP as the sole source of carbon was lost upon plasmid curing. The isoesterases Et-1–4 were absent in the cell-free extracts of the cured bacterium. The results from our studies clearly demonstrate that de-esterification is the initial step in the degradation of DMP and the genes for these esterases seem to be harbored on the plasmid in this bacterium.

Keywords: Degradation; *Bacillus* sp.; Esterase; Dimethylphthalate; Monomethylphthalate; Plasmid

1. Introduction

Phthalate and phthalate esters are industrially important chemicals, which are widely used in the manufacture of plastics, insect repellents, synthetic fibers, and cosmetics [1–5]. Phthalate esters are often discharged by the paper and plastic industries [5,6] into the environment and they have been often detected at relatively high level in the ecosystem, hence contributing to environmental pollution. Six phthalate esters: dimethylphthalate (DMP), diethylphthalate (DEP), di-*n*-propylphthalate, di-*n*-butylphthalate, di-2-ethylhexylphthalate (DEHP), and di-*n*-octylphthalate, of the 18–20 that are produced commercially are designated priority pollutants by the United States Environmental Protection Agency [1].

In nature, one of the great contributions of microorganisms to the environment is mineralization of industrial pollutants, many of which are complex organic compounds [7,8]. Several microorganisms have been isolated from the environment that have the ability to metabolize these pollutants. These include *Bacillus*, *Pseudomonas*, *Micrococcus*, *Moraxella* and *Comamonas* spp. [8–12], which convert phthalates and phthalate esters into protocatechuic acid. Earlier reports revealed that the microorganisms utilize an aerobic pathway for phthalate degradation and the major enzymes which have been identified to catabolize phthalates include phthalate oxygenase, phthalate dioxygenase, phthalate dehydrogenase, and phthalate decarboxylase [9,11,12]. Furthermore, the reports also indicate that the enzymes which mediate the catabolic pathway of phthalates are plasmid-encoded genes in some microorganisms [9,10,13,14]. However, in some cases the initial step in the degradation of phthalate esters seems to be a de-esterification reaction by specific esterases, to give rise to phthalate anions and alcohol. Kurane et al. [11] reported the involvement of esterases from *Nocardia erythropolis* hydrolyzing DEHP to phthalic acid. Recently Cartwright et al. [15] showed the existence of an alternative pathway for the degradation of DEP in the presence of methanol in a soil microbial community. In this study, we report the involvement of isoesterases in the degradation of DMP and present evidence to show that these enzymes are encoded by the genes located on the 60-kb plasmid in the *Bacillus* sp.

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2. Materials and methods

2.1. Bacterial strain and growth conditions

The *Bacillus* sp. used in this study was isolated by the selective enrichment method and maintained in this laboratory [16]. This bacterium is capable of degrading DMP completely. The organism was initially grown on a mineral salts medium amended with 0.2% (v/v) DMP before autoclaving. The flasks were inoculated with 5% inoculum and incubated at 37°C on an orbital shaker at 160 rpm for 4–6 days.

2.2. Preparation of cell-free extracts

The DMP-grown cells at mid-exponential phase ($OD_{660} = 1.24$) were harvested by centrifugation at $5000 \times g$ for 5 min at 4°C. The pellet was washed twice with 50 mM potassium phosphate buffer pH 7.0 and resuspended in 10 volumes of the same buffer. The cell suspension was ultrasonicated for 3 min at 4°C in six pulses. The ultrasonicated cell suspension was centrifuged at $30\,000 \times g$ for 20 min at 4°C. The supernatant was used as cell-free enzyme source for further studies.

2.3. Native PAGE and activity stain for esterase

The protein (100 μg) was resolved on a 10% polyacrylamide gel and electrophoresed at 4°C with 100 V [17]. Esterase staining was carried out by incubating the gel for 20 min at 37°C in substrate buffer mixture. The mixture contained 0.5% (w/v) fast blue RR salt and α-naphthyl acetate (5.38 mM initially dissolved in 0.5 ml of acetone) in 50 mM Tris–HCl buffer (pH 7.0).

2.4. Isolation of isoesterases from native gel

After preparative gel electrophoresis, a strip of the gel was cut and stained for its esterase activity as described earlier. The gel strips corresponding to esterase bands were cut, suspended in three volumes of 50 mM Tris–HCl buffer pH 7.0, and homogenized in a glass tissue homogenizer. The supernatant was collected after centrifugation at $5000 \times g$ at 4°C for 10 min, lyophilized, stored at −20°C, and used for esterase activity whenever needed. Protein estimation was carried out by measuring the absorbance at 280 nm using bovine serum albumin as standard [18].

2.5. Assay for esterase activity using p-nitrophenyl acetate (pNPA) as substrate

The reaction mixture containing 0.75 ml of 0.5 mM potassium phosphate buffer, pH 7.0 with an appropriate amount of enzyme in a total volume of 1.0 ml was incubated at 37°C for 10 min [19]. The reaction was terminated by the addition of 100 μl of 0.2 N NaOH. One unit of enzyme activity was defined as a 0.01 increase in OD_{405} under the standard assay conditions. Specific activity was expressed in units per min per mg protein under assay conditions as described above.

2.6. Assay system for degradation of DMP and monomethylphthalate (MMP)

The reaction mixture contained 60 μM of DMP or MMP in 50 mM potassium phosphate buffer, pH 7.0 in a final reaction volume of 1.0 ml and incubated with an appropriate amount of enzyme for 30 min at 37°C with suitable controls. The residual DMP/MMP was extracted with ethyl acetate and quantified by measuring the absorbance at 274 nm as described by Karegoudar et al. [8]. The specific activity was expressed in μM of DMP/MMP hydrolyzed per min per mg protein.

2.7. TLC fractionation of DMP and its hydrolyzed products

After the enzymatic hydrolysis of DMP and MMP by the esterases, the hydrolysate was acidified to pH 2.0 with 4 N HCl, extracted with ethyl acetate, and the organic layer again extracted with 5% $NaHCO_3$. The aqueous layer containing acidic compounds was extracted, dried over Na_2SO_4, concentrated, and chromatographed on silica gel TLC plates along the authentic, DMP, MMP, and phthalic acid using solvent systems: (A) benzene:acetic acid (9:1), (B) benzene:acetic acid (9:2). The spots on the TLC plate were visualized by developing in an iodine chamber and the R_f values were calculated.

2.8. Screening and curing of plasmid

The DMP-grown cells at mid-exponential phase were

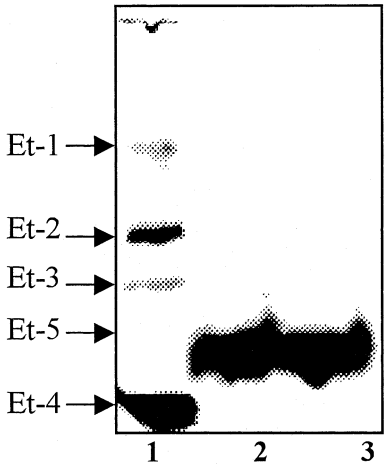

Fig. 1. Isoesterase pattern from cell-free extract of *Bacillus* sp. Lane 1: cell-free extract from wild *Bacillus* sp. grown on DMP; lane 2: cell-free extract from plasmid-cured cells by ethidium bromide treatment; lane 3: cell-free extract from plasmid-cured cells by repeated subculturing on LB.

Table 1
Specific activities of polyacrylamide gel-eluted enzymes from cell-free extracts of *Bacillus* sp. grown on DMP

Esterase	Substrate[a]		
	*p*NPA[b]	MMP[c]	DMP[c]
Et-1	146	0.14	1.360
Et-2	157	0.15	0.075
Et-3	136	0.19	0.072
Et-4	247	0.16	2.025
Et-5	002	0.01	0.001

[a]Each value is the average of triplicate determinations.
[b]Units per min per mg protein at 37°C and under standard assay conditions.
[c]μmol of DMP/MMP hydrolyzed per min per mg protein under standard assay conditions.

Fig. 2. Agarose gel electrophoresis of plasmid isolated from the *Bacillus* sp. Lane 1: control cells grown on DMP as sole source of carbon; lane 2: ethidium bromide-cured *Bacillus* sp.; lane 3: plasmid cured by repeated subculturing on LB medium.

pelleted by centrifugation and washed with GTE (glucose–Tris–EDTA) buffer. The pellet was resuspended in GTE buffer and the plasmid was isolated by the alkali lysis method [17]. The aqueous phase was washed with phenol–chloroform, and plasmid DNA was precipitated by adding two volumes of ethanol at room temperature. The precipitated DNA was recovered by centrifugation and subjected to electrophoresis after redissolving the DNA pellet in TE (10 mM Tris, 1 mM EDTA pH 8.0) on 0.8% agarose gel.

Two methods were tried to cure the plasmid from *Bacillus* sp. to check whether the cured organism retains the ability to utilize DMP as the sole source of carbon. In the first method, spontaneous loss of the above phenotype was tested by growing the organism on LB agar up to five or six subcultures followed by inoculation of the culture on agar plates containing 0.2% DMP. In the second method, the bacterium was grown in LB broth in the presence of ethidium bromide [14].

3. Results

Attempts were made to identify the esterases that hydrolyzed DMP by native PAGE. The gel pattern showed four different esterases designated Et-1, Et-2, Et-3, and Et-4 (Fig. 1, lane 1). These isoesterases Et-1–4 were isolated from the native polyacrylamide gel and activity was measured using *p*NPA, DMP, and MMP as substrates. Et-4 showed a much higher hydrolytic activity than Et-2 and Et-3, followed by Et-1 (with DMP as substrate). Et-3, although it had low *p*NPA hydrolysis activity, showed a preference towards MMP hydrolysis (Table 1). TLC analysis showed the hydrolyzed products of DMP and MMP having the R_f and λ_{max} values of these products corresponded to those of authentic MMP and phthalic acid (Table 2).

3.1. Characterization and curing of plasmid

Screening of this strain for extra chromosomal DNA demonstrated the presence of a single megaplasmid of around 60 kb. The plasmid-cured *Bacillus* sp. did not reveal any plasmid (Fig. 2). Plasmid-curing experiments revealed that the cells grown on LB agar (after five generations) and ethidium bromide lost the capacity to grow on minimal agar medium containing DMP as the sole carbon source (Fig. 3). Further, the cell-free extract from the plasmid-cured *Bacillus* cells did not show the presence of isoesterases Et-1–4, as observed in the cells grown on DMP

Table 2
TLC analysis of the hydrolyzed product of DMP by Et-1–4

Compound	R_f values in solvent systems[a]									
	A					B				
	Isolated metabolites[b]				Standards	Isolated metabolites[b]				Standards
	Et-1	Et-2	Et-3	Et-4		Et-1	Et-2	Et-3	Et-4	
MMP	0.28	0.28	0.28	0.29	0.30	–	0.37	0.37	0.35	0.36
Phthalic acid	0.05	0.04	0.06	0.06	0.06	0.30	0.30	0.29	–	0.30

Solvent systems A and B are as described in Section 2.
[a]Each value is the average of duplicate determinations.
[b]Isolated metabolites after the incubation of isoesterases Et-1–4 with DMP as substrate.

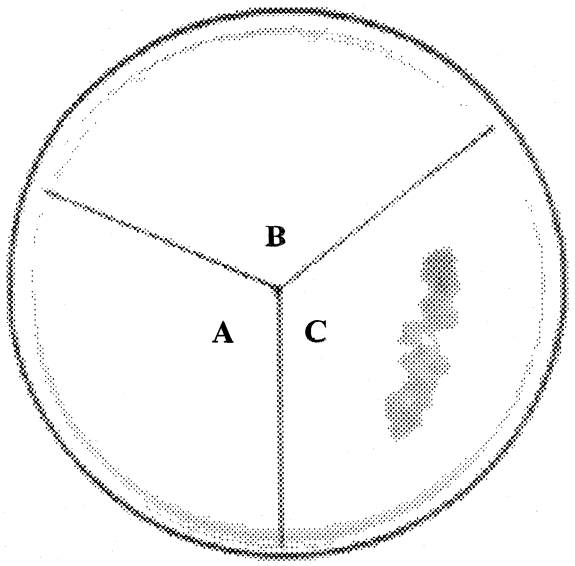

Fig. 3. Growth of *Bacillus* sp. on minimal agar plates containing 0.2% (v/v) DMP. The Petri dish contains three sectors: inoculated with the bacterium grown on (A) LB agar up to the fifth generation; (B) ethidium bromide-treated cells and (C) grown on DMP agar. Sectors A and B show no growth.

as the sole source of carbon. However, a new esterase isoform, Et-5, was observed which was different from Et-1–4 (Fig. 1, lanes 2 and 3). Further, Et-5 did not show esterolytic activity with DMP as substrate.

4. Discussion

Microorganisms are the only known organisms capable of completely degrading phthalate compounds. Most often these microorganisms belong to the group of *Pseudomonas*, *Aspergillus*, *Micrococcus*, and *Bacillus* sp. and are found to be associated with the degradation of these pollutants [8,13,20].

The primary step in the catabolic pathway for degradation of phthalate esters and their derivatives in some microorganisms seems to be mediated by de-esterification reaction. *Pseudomonas pseudoalcaligenes* de-esterifies di-*n*-butylphthalate to mono-*n*-butylphthalate and phthalic acid [21]; *N. erythropolis* hydrolyzes diethyl-hexylphthalate to phthalic acid [11]. However, Cartwright et al. [15] showed that the initial step is not necessarily de-esterification. However, the results of our study on the mineralization of DMP by *Bacillus* sp. seem to involve an initial de-esterification step before it is metabolized to TCA intermediates. Further, analysis of the esterases from this bacterium clearly demonstrates the existence of four isoesterases (Et-1–4) and two of them have very good DMP-hydrolyzing ability (Table 1). Although the relative specific activity of isoesterases in utilizing *p*NPA and MMP as substrate seems to be almost the same for these isoesterases, they differ in de-esterification of DMP. The results clearly indicate that Et-4, followed by Et-1, hydrolyzes DMP with high specific activity as compared to Et-2 and Et-3. Further, it was observed from these results that Et-1 and Et-4 might prefer diesters as compared to monoesters as substrates.

Furthermore, our results demonstrate the presence of a megaplasmid of molecular size 60 kb. The results clearly revealed that the plasmid-cured cells lost their ability to grow on DMP. The isoesterases, especially Et-1 and Et-4, have a higher affinity for DMP. This showed that the cured strain lacks these isoesterases and also failed to utilize DMP (in vivo). However, a new endogenous esterase isoform, Et-5, was found in cured cells which is not expressed in the presence of DMP as the sole carbon source. This is probably required to meet the normal cellular functions. From these results we suggest that the isoesterases (Et-1–4) are probably encoded by the plasmid and mediate the initial step of de-esterification, in the mineralization of DMP.

It has been recently reported that the degradation product of 4-methylphthalate by *Pseudomonas cepacia* harbored a plasmid in which the *mopB* gene encodes 4-methylphthalate permease. This has been implied to be involved in 4-methylphthalate catabolism [13]. A similar observation was reported in *Moraxella* sp. where 60–70-kb plasmids encoding phthalate 4,5-dioxygenase and 4,5-dihydro-4,5-dihydroxyphthalate dehydrogenase are involved in *o*-phthalate catabolism [14]. Further studies on the isolation and characterization of esterase involved in the initial degradation of DMP from this bacterium are in progress.

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