Full Paper

Isolation and identification of dibutyl phthalate-degrading bacteria from hydrospheres in Tokyo

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(Received September 24, 2008; Accepted March 13, 2009)

Dibutyl phthalate (DBP) is used widely as a plasticizer and is thought to negatively affect various organisms. To isolate and investigate DBP-degrading bacteria from hydrospheres in Tokyo, strains were selected on YNB medium containing DBP as the sole carbon source, and candidate strains were identified by zones of clearing around the colonies. Degradation of DBP by the strains was subsequently measured with HPLC, and bacterial identification was accomplished using 16S rDNA sequences. Nineteen strains of DBP degraders were isolated from activated sludge in a sewage treatment plant, from Tokyo Bay, and from the Takahama Canal. These strains degraded 16.8%–88.0% of DBP (0.1%, v/v) for 2 weeks and were identified as several species of *Acinetobacter*, as well as *Tsukamurella tyrosinosolvens*, *Ochrobactrum anthropi*, and *Staphylococcus saprophyticus*. Commercially available strains of *Acinetobacter* were also found to degrade DBP.

Key Words—*Acinetobacter*; biodegradation; dibutyl phthalate; hydrospheres

Introduction

Phthalate esters are widely used as plasticizers of polyvinyl chloride. Approximately 4.3 million tons per year of phthalate esters are produced worldwide, which has been at a constant level for 20 years (Peijnenburg and Struijs, 2006). Harmful effects (toxicity, growth suffocation) of dibutyl phthalate (DBP) on various organisms have been reported (Hallmark et al., 2007; Huang et al., 2006; Jonsson and Baun, 2003). Besides, the use of DBP in cosmetics is prohibited in the European Union (Mann, 2004). Additionally, DBP has been detected in several hydrospheres (for example; seawater, river sediment, and the yellow river in China) in recent years (Peijnenburg and Struijs, 2006;

Sha et al., 2007; Yuan et al., 2002).

There have been some reports describing the results of searches for DBP-degrading bacteria in nature (Chao et al., 2006; Chauret et al., 1995; Xu et al., 2005). However, there have been few reports concerning the isolation of DBP-degrading bacteria from urban hydrospheres. Previously, we observed the degradation of organic compounds using a bacterial strain isolated from the hydrosphere in Tokyo (Fujii et al., 2000a, b, 2001). Therefore, in an effort to identify additional strains of DBP-degrading bacteria, we conducted a search for such bacteria in Tokyo. We describe here the successful identification and isolation of DBP-degrading bacteria, and we compare the degradation ability of these naturally isolated strains with that of commercially available strains.

Materials and Methods

Isolation of a DBP degrader. Water samples were collected from the first and fourth activated sludge

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tanks and from sludge drainage water in a sewage treatment plant, from Tokyo Bay, and from the Taka-hama Canal. One hundred microliters of each water sample was spread onto a YNB-DBP agar plate containing 0.67% Yeast Nitrogen Base without amino acids (BD, NJ, USA), 0.1% DBP (Wako, Osaka, Japan), and 2% agar (Kokusan Chemical, Tokyo, Japan). The DBP was diffused into the entire medium with an Ultra-sonic processor VP-60 (Taitec, Saitama, Japan). After incubation at 25°C for 1–2 weeks, colonies exhibiting cleared zones in the medium were selected as DBP degraders and were stored at 10°C. The colony-forming units (cfu) of bacterial cells forming colonies on nutrient agar (NA) (Eiken Chemical, Tokyo, Japan) and of clear-zone-forming cells were counted for the activated sludge water sample.

DBP degradation test. The clear-zone-forming strains were inoculated into a 100-ml flask with 10 ml of liquid medium containing 0.67% Yeast Nitrogen Base without amino acids (BD), 0.1% DBP (Wako), and 0.3% Casamino acids (Wako). Cultures were cultivated at 25°C/120 rpm in the dark for 2 weeks. After incubation, 50 ml of acetonitrile (Wako) was added to the cultivation medium to dissolve DBP, and the mixture was shaken for 2 min with a Branson 5210 ultrasonic cleaner (Yamato Scientific, Tokyo, Japan). Forty milliliters of distilled water was added, and the culture was filtered (0.45- μ m pore size, Juji Field, Tokyo, Japan). Samples were stored at –30°C until HPLC analysis.

Measurement of DBP. DBP was measured with an HPLC system (Tosoh, Tokyo, Japan). A LiChroCART 125-4 column (Merck, Darmstadt, Germany) was used for separation at 28°C. The mobile phase was water : acetonitrile (Wako)=80 : 20 up to 5 : 95 in 30 min. DBP and its microbial metabolites were monitored at 224 nm with a UV detector. Under these chromatographic conditions, a peak of DBP was obtained at 22 min, and the concentration detection limit was 2.5 μ l/L. Correlation of the calibration line was 0.99. The degradation ratio was defined as the ratio of degraded DBP to that of the initial amount of DBP. DBP, phthalic acid, and protocatechuic acid (Wako) were used as chemical standards.

Identification of isolated strains. Identification of isolates was performed by analyzing 16S rDNA partial sequences as follows: Nucleic acids were extracted from the cells using Isoplant II (Nippon Gene, Toyama, Japan) and were then subjected to PCR by using the

27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3') primer set (Lane, 1991). PCR was performed with a Thermal Cycler Dice TP650 (TaKaRa Bio, Shiga, Japan) under the following conditions: 95°C: 2 min, 30 cycles (95°C: 1 min, 55°C: 1 min, 72°C: 2 min), 72°C: 2 min. The PCR products were purified using ExoSAP-IT (GE Healthcare, Buckinghamshire, England) and then sequenced using a Big-Dye terminator cycle sequencing kit v3.1 (Applied Biosystems, CA, USA) with the 27F primer and Eu-b338R (5'-GCTGCCTCCCGTAGGAGT-3') primer set (Aminov et al., 2006). The products were purified using the DyeEx 2.0 spin kit (Qiagen, Tokyo, Japan) and analyzed using an ABI Prism 310 Genetic Analyzer (Applied Biosystems). All processes were performed according to the manufacturer's instructions. The BLASTN program (Altschul et al., 1997) was then used to determine homology with other organisms. The 16S rDNA sequence data determined in this study were registered in the DNA Data Base of Japan (DDBJ).

Organisms. DBP degradation tests (described above) were performed on purchased strains that were the same species as those we identified from the environment. Each microorganism used in the DBP degradation tests was obtained from the NITE Biological Resource Center (NBRC) or Gifu University (GTC) in Japan. These strains were tested for clear-zone-forming ability on DBP-NB agar plates containing 0.1% DBP, 1.8% nutrient broth (Eiken Chemical), and 2% agar (Kokusan Chemical). After incubation for 1 week at 25°C, clear zones around the colonies were confirmed.

Results and Discussion

The number of clear-zone-forming cells in activated sludge

The number of clear-zone-forming cells and colony-forming cells on NA are indicated in Table 1. Many clear-zone-forming cells were observed from bacteria isolated from both the first and fourth activated sludge tanks, but no more than 10 cells/ml were observed in the influx water and the sludge drainage water. These results indicate that there were many DBP-degrading cells in the activated sludge, which confirms previous studies about biodegradation of DBP using activated sludge (Amir et al., 2005; Roslev et al., 2007; Wang, 2004). Therefore, we suspect that samples of activated sludge from other locations also would likely contain

Table 1. Population of DBP-degradable bacteria isolated from a sewage treatment plant.

	Sample origin			
	Influx water	First activated sludge tank	Fourth activated sludge tank	Sludge drainage water
Colony-forming cells (cfu/ml)	$4.6 \pm 0.6 \times 10^5$	$1.2 \pm 0.1 \times 10^7$	$3.9 \pm 0.2 \times 10^6$	$3.4 \pm 1.9 \times 10^3$
Clear-zone-forming cells (cfu/ml)	<10	$1.5 \pm 0.7 \times 10^4$	$6.0 \pm 2.0 \times 10^3$	<10

Each value is an average $\pm$ range of two experiments.

Table 2. DBP degradation ratios and strain identification of isolated strains.

Strain no. (DDBJ accession no.)	DBP degradation ratio ^a (%)	Closest organism (DDBJ accession no.)	Homology (%)
TC5 (AB453189)	34.0 $\pm$ 12.9	<i>Ochrobactrum anthropi</i> (EU275247)	100
TC7 (AB453190)	44.4 $\pm$ 6.2	<i>Acinetobacter</i> sp. (EF577080)	99.6
TC18 (AB453191)	37.8 $\pm$ 8.1	<i>Ochrobactrum anthropi</i> (U88443)	100
TC19 (AB453192)	41.8 $\pm$ 1.9	<i>Acinetobacter</i> sp. (EF577080)	99.6
TC21 (AB453193)	50.5 $\pm$ 4.1	<i>Acinetobacter</i> sp. (EF577080)	99.6
TB40 (AB453194)	22.2 $\pm$ 1.4	<i>Acinetobacter junii</i> (AM184279)	100
TB47 (AB453195)	21.1 $\pm$ 2.0	<i>Acinetobacter</i> sp. (EU000448)	100
TB52 (AB453196)	30.7 $\pm$ 3.7	<i>Acinetobacter</i> sp. (EU000455)	100
1AS83 (AB453197)	16.8 $\pm$ 1.6	<i>Acinetobacter</i> sp. (EU000451)	100
1AS99 (AB453198)	25.4 $\pm$ 1.0	<i>Acinetobacter baumannii</i> (Z93435)	100
1AS113 (AB453199)	40.5 $\pm$ 7.9	<i>Acinetobacter</i> sp. (EU382216)	100
1AS116 (AB453200)	45.3 $\pm$ 10.7	<i>Acinetobacter</i> sp. (EU240202)	100
1AS117 (AB453201)	27.4 $\pm$ 1.2	<i>Acinetobacter johnsonii</i> (AM184271)	97.8
1AS123 (AB453202)	36.1 $\pm$ 2.0	<i>Acinetobacter</i> sp. (EF494200)	99.6
4AS73 (AB453203)	54.7 $\pm$ 5.9	<i>Acinetobacter</i> sp. (DQ421391)	100
4AS75 (AB453204)	26.6 $\pm$ 2.4	<i>Acinetobacter</i> sp. (Z93436)	100
4AS76 (AB453205)	17.5 $\pm$ 1.4	<i>Acinetobacter</i> sp. (EF429004)	98.6
4AS126 (AB453206)	88.0 $\pm$ 11.6	<i>Tsukamurella tyrosinosolvens</i> (AY238514)	100
4AS127 (AB453207)	78.4 $\pm$ 4.8	<i>Staphylococcus saprophyticus</i> (Z26902)	100

^an=3, mean $\pm$ SEM.

The strain names indicate their sources; TC=Takahama Canal, TB=Tokyo Bay, 1AS=first activated sludge, 4AS=fourth activated sludge.

DBP-degrading bacteria.

Degradation ratios of DBP from isolated strains and strain identification

The degradation ratios of DBP from 19 isolated strains and their respective identities are listed in Table 2. These strains degraded 16.8%–88% of DBP and had 97.8%–100% homology with several species of *Acinetobacter*, as well as *Tsukamurella tyrosinosolvens*, *Ochrobactrum anthropi*, and *Staphylococcus saprophyticus*. The presence of these genera/species in hydrospheres was consistent with previous studies,

for example, *Acinetobacter* (Barberio and Fani, 1998), *Tsukamurella* (Nam et al., 2004), *Staphylococcus saprophyticus* (Xin and Wang, 2007), and *Ochrobactrum anthropi* (Pesce and Wunderlin, 2004). In particular, *Acinetobacter* was found in all of the water samples and accounted for 15 of the 19 isolated strains. Most strains of *Acinetobacter* can grow in simple mineral medium containing a single carbon source (Juni, 1984). There has been a report that *Acinetobacter* spp. account for 75% of isolates when nonylphenol ethoxylates are used as the sole carbon source for bacteria isolated from activated sludge (Barberio and Fani,

1998). Therefore, we suppose the YNB-DBP agar selected the *Acinetobacter* strains. *Acinetobacter* strains with DBP degradation ability have been isolated previously from river water in Japan (Hashizume et al., 2002). Some *Acinetobacter* strains have been reported to degrade organic compounds (Juni, 1984). Therefore, we propose that *Acinetobacter* spp. exist as DBP degraders in the investigated hydrospheres. Otherwise, the isolation of *Tsukamurella*, *Staphylococcus*, and *Ochrobactrum* as DBP degraders is a novel finding of this study.

After the degradation tests, peaks with retention times of 1.0 min and 1.3 min appeared during HPLC of all 19 samples. These retention times corresponded with phthalic acid and protocatechuic acid, respectively. Phthalic acid (Wang et al., 1999; Xu et al., 2005) and protocatechuic acid (Gu et al., 2005; Quan et al., 2005) have been reported as metabolites of DBP or other phthalic esters, respectively. Therefore, the metabolites produced by the isolated strains may be phthalic acid and protocatechuic acid. However, it will require more precise analysis to definitively identify the metabolites.

DBP degradation by commercially available strains

The degradation ratios of DBP by purchased strains are listed in Table 3. All of the tested *Acinetobacter* strains degraded 23.2%–39.3% of DBP and formed clear zones around the colonies on DBP-NB agar plates. These results suggest that DBP-degrading ability is a common characteristic of *Acinetobacter* species. However, although the purchased *Staphylococcus saprophyticus* strain slightly degraded DBP as measured by HPLC, clear zone formation was not observed. It may be that the lack of clear zone formation in this strain was caused by a low degradation ratio.

The purchased strain *Ochrobactrum anthropi* didn't degrade DBP, unlike the isolated strains of the same species (TC5 and TC18). Therefore, these strains may have acquired DBP-degrading ability from movable genes such as transposons.

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Table 3. DBP degradation with purchased strains in solid and liquid media.

Strain no.	Clear zone formation ^a	DBP degradation ratio ^b (%)
<i>Acinetobacter junii</i> GTC 00441	+	37.6±2.1
<i>Acinetobacter johnsonii</i> GTC 11743	+	23.2±2.2
<i>Acinetobacter baumannii</i> GTC 11741	+	25.8±2.3
<i>Acinetobacter</i> sp. NBRC100985	+	39.3±1.8
<i>Staphylococcus saprophyticus</i> NBRC 102446	–	1.8±0.9
<i>Ochrobactrum anthropi</i> NBRC 15819	–	Not degraded

To determine the DBP degradation ratio and clear zone formation, bacterial strains were grown in YNB-DBP liquid media (containing 0.3% casamino acid) and NB-DBP solid media, respectively. *Tsukamurella tyrosinosolvens* was not purchased.

^aClear zone formation was tested on NB-DBP plates. +, confirmed; –, not confirmed. ^b*n*=3, mean ± SEM.

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