Bull. Environ. Contam. Toxicol. (2004) 72:911–915

DOI: 10.1007/s00128-004-0330-3

Bulletin of Environmental Contamination and Toxicology

# Identification of the Phthalate-Degrading Bacteria Isolated from Phthalate-Contaminated Soil and Characterization of Their Phthalate Substrate Specificity

S. H. Ahn,[1] J. H. Lee,[2] Y. P. Hong,[3] M. K. Kim[1]

[1] School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea
[2] Department of Food Science and Biotechnology, Kyonggi University, Suwon 442-760, Korea
[3] Department of Preventive Medicine and Community Health, Chung-Ang University, Seoul 156-756, Korea

Received: 15 January 2003/Accepted: 21 January 2004

Phthalate esters are used as additives to improve the mechanical properties of plastics, particularly that of flexibility. In addition, they are used in packaging materials and containers of insecticides and cosmetics. The number of these compounds is increasing owing to the increase in different types of synthetic resin products (Lee and Lee, 2001). However, because phthalate plasticizers are not covalently bound to the resin, they are able to easily migrate into the environment.

Phthalate ester has the basic structure of an esterified benzenedicarboxylic acid with two alkyl chains. Decomposition of phthalate esters in the environment mainly occurs through microbiologically mediated processes that follow a series of stages common for all phthalate esters (Ribbons *et al.*, 1984; Staples *et al.*, 1997).

We have isolated bacteria that use phthalates as a carbon source for energy. The bacteria were recovered from soil highly contaminated with phthalate. The bacteria were subsequently identified using 16S ribosomal DNA (rDNA) sequencing, and their substrate specificity was compared.

## MATERIALS AND METHODS

Soil was sampled near Anyangcheon, Korea, a region found to be highly polluted with phthalate esters (Ahn *et al.*, 2002). The sampling was performed at a depth of 10 cm from the soil surface.

Each soil sample was suspended in 0.9% sterilized NaCl solution. One ml of the supernatant was added into 30 ml each of PA and DEHP minimal liquid media, cultured by shaking at 250 rpm for 3 d at 28℃, serially diluted, and spread on PA and DEHP minimal agar media for 2 d at 28℃, respectively. Well-grown colonies were suspended in 0.9% sterilized NaCl solution, serially diluted, and spread on the same agar media originally grown. Mature single colonies on both media were selected as the bacteria degrading PA and DEHP.

Phthalic acid (PA) or bis(2-ethylhexyl) phthalate (DEHP) was added as the carbon source at either 5 or 25 mM into the minimal medium consisting of 1.0 g $NH_4Cl$, 2.0 g $KH_2PO_4$, 0.5 g $MgSO_4$, 0.5 g KCl, 0.1 g $CaCl_2{\cdot}2H_2O$, 0.1 g EDTA, 0.01 g $FeCl_3{\cdot}6H_2O$, 5 $\mu$g $CuSO_4{\cdot}5H_2O$, 1 $\mu$g $H_3BO_3$, and 1.6 $\mu$g $MnCl_2{\cdot}4H_2O$ per liter of distilled water. pH was adjusted to 7.0 using 1 N NaOH (Lee, 1993; Lee and Lee, 2001). All chemicals mentioned above were purchased from Sigma (St. Louis, MO), and their purities were at least 99% except for $FeCl_3{\cdot}6H_2O$ (minimum 98%) and NaOH (minimum 98%).

*Correspondence to:* M. K. Kim

Bacterial cells were grown in 10 ml LB broth at 30℃ overnight and harvested by centrifugation. Bacterial genomic DNA was isolated according to the method of Sambrook and Russell (2001).

PCR was carried out to amplify 16S rDNA of cultured phthalate-degrading bacteria. PCR primers were designed from the 100% sequence-conserved regions of 10 different bacteria [5 Gram-positive (*Bacillus halophilus*, *Corynebacterium renale*, *Lactobacillus psittaci*, *Staphylococcus piscifermentans*, and *Streptococcus pneumoniae*) and 5 Gram-negative (*Comamonas nitrativorans*, *Escherichia coli*, *Helicobacter canis*, *Pseudomonas fluorescence*, and *Vibrio scophthalmi*) bacteria], based on the sequence alignment using San Diego Supercomputer Center (SDSC) Biology Workbench web site. The sequences of upstream and downstream primers were 5′ ccagactcctacgggaggcag 3′ and 5′ ctcacgacacgagctcacgac 3′, respectively. Denaturation was done for 1 min at 94℃, annealing for 1 min at 63℃, and polymerization for 1 min at 72℃. The PCR products, purified using a QIAquick PCR purification kit (QIAGEN, Valencia, CA), were ligated to pGEM-T Easy Vector (Promega, Madison, WI) and sequenced at Green Gene Biotech (Yongin, Korea). The sequence alignment was performed using BLAST program [National Center for Biotechnology Information (NCBI)].

Bacterial isolates, tentatively identified based on the sequencing of the 16S rDNA region, were first grown in minimal liquid media containing 25 mM succinate as a carbon source until the optical density at 600 nm ($OD_{600}$) reached about 0.5. They were then streaked on minimal agar plates containing 25 mM each of PA and DEHP as carbon sources and incubated at 28℃ for 1 wk. Equal numbers of bacterial isolates were also inoculated into PA- and DEHP-containing minimal liquid media and grown at 30℃ for 1 mon. In solid and liquid cultures, bacterial growth was decided based on the size of colonies and by measuring $OD_{600}$, respectively.

## RESULTS AND DISCUSSION

Five and four colonies were isolated from pure cultures in PA- and DEHP-containing minimal media, respectively. The isolates from 5 mM PA medium were designated as PA 5-1, 5-2, 5-3, 5-4, and 5-5, that from 5 mM DEHP medium was designated DEHP 5-1, and those from 25 mM DEHP medium were designated DEHP 25-1, 25-2, and 25-3 (Table 1).

**Table 1.** Tentative identification of phthalate-degrading bacteria based on 16S rDNA sequence comparison with NCBI database search.

| Isolated colony | Taxonomy | Identity |
|---|---|---|
| PA 5-1 | *Comamonas testosteroni* | 747/749 bp (99%) |
| PA 5-2* | *Comamonas testosteroni* | 748/749 bp (99%) |
| PA 5-3 | *Comamonas testosteroni* | 748/749 bp (99%) |
| PA 5-4* | *Comamonas testosteroni* | 748/749 bp (99%) |
| PA 5-5** | *Acinetobacter* sp. DVL00/879 | 735/749 bp (98%) |
| DEHP 5-1** | *Acinetobacter* sp. DVL00/879 | 735/749 bp (98%) |
| DEHP 25-1*** | *Acinetobacter haemolyticus* | 748/749 bp (99%) |
| DEHP 25-2*** | *Acinetobacter haemolyticus* | 748/749 bp (99%) |
| DEHP 25-3 | *Pseudomonas tolaasii* | 745/748 bp (99%) |

Asterisks indicate same sequences.

The isolated bacteria were tentatively identified as the genera *Comamonas*, *Acinetobacter*, and *Pseudomonas* based on over 98% sequence homology of the PCR-amplified 16S rDNA regions of the isolated bacteria with those registered on NCBI database. Four among five isolates from PA minimal media belonged to *Comamonas testosteroni*, whereas the remaining one belonged to *Acinetobacter* sp. DVL00/879. Two isolates from DEHP minimal media were identified as *Acinetobacter haemolyticus*, and the other two as *Acinetobacter* sp. DVL00/879 and *Pseudomonas tolaasii*. Sequences of the isolates PA 5-2 and 5-4, those of PA 5-5 and DEHP 5-1, and those of DEHP 25-1 and 25-2, respectively, were in perfect agreement.

Phthalate substrate specificities of the bacteria are shown in Table 2. Results revealed *C. testosteroni* preferred PA to DEHP as the carbon source, whereas *Acinetobacter* spp. and *P. tolaasii* used both at similar frequencies. The fact that *Acinetobacter* sp. DVL00/879 was isolated from both PA and DEHP media is consistent with these results. It is likely that *C. testosteroni* prefers benzene ring opening to alkyl chain cleavage.

**Table 2.** Substrate specificity of phthalate-degrading bacteria.

| Isolated colony | Taxonomy | PA | | DEHP | |
|---|---|---|---|---|---|
| | | Solid | Liquid | Solid | Liquid |
| PA 5-1<br>PA 5-2<br>PA 5-3<br>PA 5-4 | *Comamonas testosteroni* | ++++ | +++ | - | - |
| PA 5-5<br>DEHP 5-1 | *Acinetobacter* sp. DVL00/879 | +++ | +++ | ++ | +++ |
| DEHP 25-1<br>DEHP 25-2 | *Acinetobacter haemolyticus* | ++ | +++ | ++ | ++ |
| DEHP 25-3 | *Pseudomonas tolaasii* | + | ++ | + | ++ |

In cases of solid culture, average colony diameters are presented: ++++, > 2.0 mm; +++, 1.5~2.0 mm; ++, 1.0~1.5 mm; +, 0.5~1.0 mm; -, < 0.5 mm. In cases of liquid culture, ++++, $OD_{600}$ > 1.0; +++, $OD_{600}$ 0.7~1.0; ++, $OD_{600}$ 0.4~0.7; +, $OD_{600}$ 0.1~0.4; -, $OD_{600}$ < 0.1.

*Comamonas testosteroni* [formerly *Pseudomonas testosteroni* (Tamaoka *et al.*, 1987)] is known to have the ability to degrade PA (Stanier *et al.*, 1966; Wheelis *et al.*, 1967; Dennis *et al.*, 1973). However, there have been no reports that *Acinetobacter* spp. and *Pseudomonas tolaasii* degrade phthalates, even though *Acinetobacter* sp. strain ADP1 (formerly *Acinetobacter calcoaceticus*) is known to degrade benzoic acid, whose structure is similar to PA (Karlsson *et al.*, 2002).

The main cause of substrate specificity is supposed to result from the differences in the activities of enzymes involved in phthalate catabolism and the cytoplasmic membrane stability towards PA and DEHP (Cartwright *et al.*, 2000) among bacterial genera. In *Pseudomonas* spp., PA is first dihydroxylated by PA dioxygenase to give 4,5-dihydro-4,5-dihydroxyphthalic acid (DDP), which then is dehydrogenated by DDP dehydrogenase to give 4,5-dihydroxyphthalic acid (DHP), and finally decarboxylated by the key enzyme, DHP decarboxylase, to give protocatechuic acid (PCA) (Ribbons *et al.*, 1984; Lee, 1993; Lee *et al.*, 1994; Chang and Zylstra, 1998) (Figure 2). In the case of *Micrococcus* spp., dihydroxylation and dehydrogenation

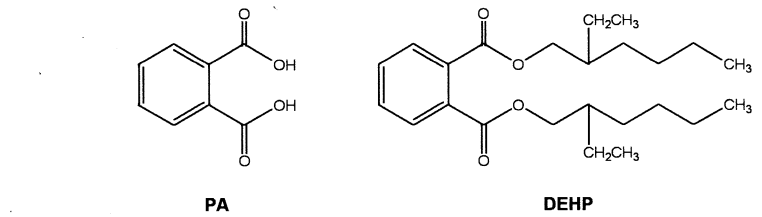


**Figure 1.** Chemical structures of phthalate derivatives as carbon sources. Abbreviations: PA, phthalic acid; DEHP, bis(2-ethylhexyl) phthalate.

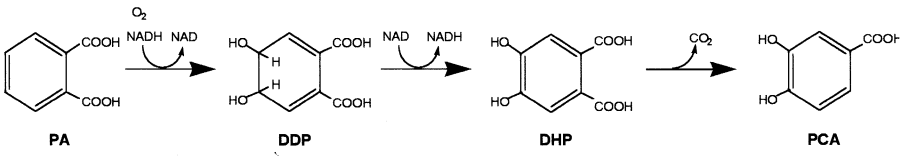


**Figure 2.** Degradation pathway of phthalic acid by *Pseudomonas* spp. Abbreviations: PA, phthalic acid; DDP, 4,5-dihydro-4,5-dihydroxyphthalate; DHP, 4,5-dihydroxyphthalate; PCA, protocatechuic acid.

occur on positions 3 and 4 of the benzene ring instead of 4 and 5 (Ribbons *et al.*, 1984). PCA dioxygenase catalyzes the opening of the benzene ring. *C. testosteroni* specifically degraded PA probably because enzymes, such as PA dioxygenase, involved in PA catabolism show high activities in the presence of PA, whereas it has relatively weak ability to degrade the alkyl chain of DEHP. However, *Acinetobacter* spp. and *P. tolaasii* degraded PA and DEHP at similar frequencies. Therefore, it is likely that enzymes involved in alkyl chain cleavage of DEHP have higher activities in cases of *Acinetobacter* spp. and *P. tolaasii* compared to that of *C. testosteroni*. These results indicate that the expression or the presence of genes involved in the phthalate metabolism varies among the soil bacterial genera.

*Acknowledgments.* This study was supported by research grants from the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (HMP-00-CH-18-0017), and from Crop Functional Genomics Center of the 21st Century Frontier Research Program funded by the Korean Ministry of Science and Technology (code CG172). Seung-Hyun Ahn was supported by the Korean Ministry of Education through the Brain Korea 21 project.

## REFERENCES

Ahn SH, Lee JH, Hong YP, Kim MK (2002) Analysis of soil contamination by phthalate ester around tributaries to the Han river. J Korean Soc Agric Chem Biotechnol 45: 97-100 (in Korean)

Cartwright CD, Thompson IP, Burns RG (2000) Degradation and impact of phthalate plasticizers on soil microbial communities. Environ Toxicol Chem 19: 1253-1261

Chang HK, Zylstra GJ (1998) Novel organization of the genes for phthalate degradation from *Burkholderia cepacia* DBO1. J Bacteriol 180: 6529-6537

Dennis DA, Chapman PJ, Dagley S (1973) Degradation of protocatechuate in *Pseudomonas testosteroni* by a pathway involving oxidation of the product of *meta*-fission. J Bacteriol 113: 521-523

Karlsson A, Beharry ZM, Eby DM, Coulter ED, Neidle EL, Kurtz DM Jr, Eklund H, Ramaswamy S (2002) X-ray crystal structure of benzoate 1,2-dioxygenase

reductase from *Acinetobacter* sp. strain ADP1. J Mol Biol 318: 261-272
Lee JH (1993) Studies on the genes involved in the phthalate metabolism in *Pseudomonas testosteroni* M4-1. Ph.D. Thesis, The University of Tokyo, Tokyo, Japan
Lee JH, Omori T, Kodama T (1994) Identification of the metabolic intermediates of phthalate by Tn5 mutants of *Pseudomonas testosteroni* and analysis of the 4,5-dihydroxyphthalate decarboxylase gene. J Ferment Bioeng 77: 583-590
Lee Y, Lee JH (2001) Degradation of phthalic acid isomers by terephthalic acid degrading bacteria isolated from Kyonggi area. Korean J Appl Microbiol Biotechnol 29: 1-5 (in Korean)
Ribbons DW, Keyser P, Kunz DA, Taylor BF, Eaton RW, Anderson BN (1984) Microbial degradation of phthalates. In: Gibson DT (ed) Microbial Degradation of Organic Compounds. Marcel Dekker, New York, pp 371-397
Sambrook J, Russell DW (2001) Molecular cloning: A laboratory manual (3rd edition). Cold Spring Harbor Laboratory Press, New York
Stanier RY, Palleroni NJ, Doudoroff M (1966) The aerobic pseudomonads: A taxonomic study. J Gen Microbiol 43: 159-271
Staples CA, Peterson DR, Parkerton TF, Adams WJ (1997) The environmental fate of phthalate esters: A literature review. Chemosphere 35: 667-749
Tamaoka J, Ha DM, Komagata K (1987) Reclassification of *Pseudomonas acidovorans* den Dooren De Jong 1926 and *Pseudomonas testosteroni* Marcus and Talalay 1956 as *Comamonas acidovorans* comb. nov. and *Comamonas testosteroni* comb. nov., with an emended description of the genus *Comamonas*. Int J Syst Bacteriol 37: 52-59
Wheelis ML, Palleroni NJ, Stanier RY (1967) The metabolism of aromatic acids by *Pseudomonas testosteroni* and *P. acidovorans*. Arch Mikrobiol 59: 302-314