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Isolation and Identification of Persistent Chlorinated Organophosphorus Flame Retardant-Degrading Bacteria[▽]

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Tris(2-chloroethyl) and tris(1,3-dichloro-2-propyl) phosphates are chlorinated persistent flame retardants that have recently emerged as environmental pollutants. Two bacterial strains that can degrade the compounds when they are the sole phosphorus sources have been isolated and identified as members of the sphingomonads. The strains can be useful for the bioremediation of environments contaminated with these compounds.

The chlorinated organophosphorus (OP) compounds tris(2-chloroethyl) phosphate (TCEP) and tris(1,3-dichloro-2-propyl) phosphate (TDCPP) are widely used as flame retardants mainly in a range of plastic foams, resins, and latexes and in the production of liquid unsaturated polyester resins, respectively (21). Their widespread use has led to their contamination of various environments, including indoor air and house dust (9, 15), surface water (2), ground water (4), rain and snow (11), and sediment (6), in concentrations ranging from ng/liter up to several μg/liter. Particularly, a higher contamination level was observed in leachates and raw water of waste disposal sites (7). Furthermore, they were also detected in drinking water (21) because they are hard to eliminate not only in the environment but also in sewage treatment plants (1). Many studies have shown several toxic effects of the compounds. TCEP has been shown to cause adverse effects on brain, liver, and kidney and on the fertility of male rats and mice (21). In addition, TCEP has produced tumors at various organ sites (19) and has also been suspected to possess carcinogenicity (10); although there are contradictory results on its carcinogenicity (3), TCEP has been shown to inhibit the expression of cell cycle regulatory proteins, DNA synthesis, and cell numbers (12). TDCPP has exhibited genotoxicity in several *in vitro* assays conducted in prokaryotic and eukaryotic cells (14) and produced some indications of carcinogenicity (21). These observations have prompted the recognition of potential ecological and human health concerns.

Many bacteria and fungi capable of degrading OP pesticides and insecticides, such as parathion and chlorpyrifos, have been discovered, isolated, and characterized (13). In contrast, few studies have been conducted on microbial degradation of chlorinated OP flame retardants, despite their persistence and potential, nonnegligible toxicity. Thus far, there has been no report of the isolation of bacteria capable of degrading TCEP and TDCPP.

We recently demonstrated that these compounds were rapidly degraded in two enrichment bacterial cultures, named 45D and 67E, obtained by using TCEP and TDCPP as sole phosphorus sources (16). We therefore attempted to isolate bacteria capable of degrading the compounds from each enrichment culture. Five hundred microliters of 45D stored at 4°C was transferred to 100 ml of A-Cl medium containing 20 μM TDCPP (Wako Pure Chemical, Japan) as the sole phosphorus source (16). Medium A-Cl is a minimal medium composed of 10 g/liter glucose, 5.22 g/liter 3-morpholinepropanesulfonic acid, 1 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.032 g of $Ca(NO_3)_2 \cdot 4H_2O$, and 10 ml of a trace element solution in 1 liter of distilled water. The final pH was 7.4. The trace element solution was composed of 500 mg of $FeSO_4 \cdot 7H_2O$, 143 mg of $MnSO_4 \cdot 2H_2O$, 22 mg of $ZnSO_4 \cdot 7H_2O$, 12 mg of $CoSO_4 \cdot 7H_2O$, 3 mg of $CuSO_4 \cdot 5H_2O$, 2.3 mg of $Na_2WO_4 \cdot 2H_2O$, and 2 mg of $Na_2MoO_4 \cdot 2H_2O$ in 1 liter of distilled water. After incubation at 30°C for 2 days with shaking at 165 rpm, cells were harvested by centrifugation at 2,500 × *g* for 5 min at room temperature, washed twice with medium lacking TDCPP, and resuspended in the same fresh medium. The cell suspension was inoculated into 100 ml of A-Cl medium containing 20 μM TDCPP as the sole phosphorus source at a final optical density at 600 nm (OD_{660}) of 0.05, and it was incubated at 30°C for 4 h with shaking. An aliquot (0.5 ml) of the culture was transferred into 4.5 ml of A-Cl medium containing 20 μM TDCPP as the sole phosphorus source in a test tube and then subsequently serially diluted by transferring 0.5 ml of the resulting culture to 4.5 ml of the same medium in a test tube. The diluted cultures were cultivated at 30°C with shaking at 165 rpm until cell growth was observed. This serial dilution cultivation was repeated several times, using the highest dilution that exhibited cell growth as the inoculum for the next cultivation. Finally, the culture was spread onto an A-Cl agar plate containing 232 μM TDCPP as the sole phosphorus source and incubated at 30°C. A single colony was picked from the plate and named strain TDK1. To isolate that from the enrichment culture 67E, the culture stored at 4°C was diluted appropriately with saline. An aliquot (0.1 ml) of the dilution was spread onto an

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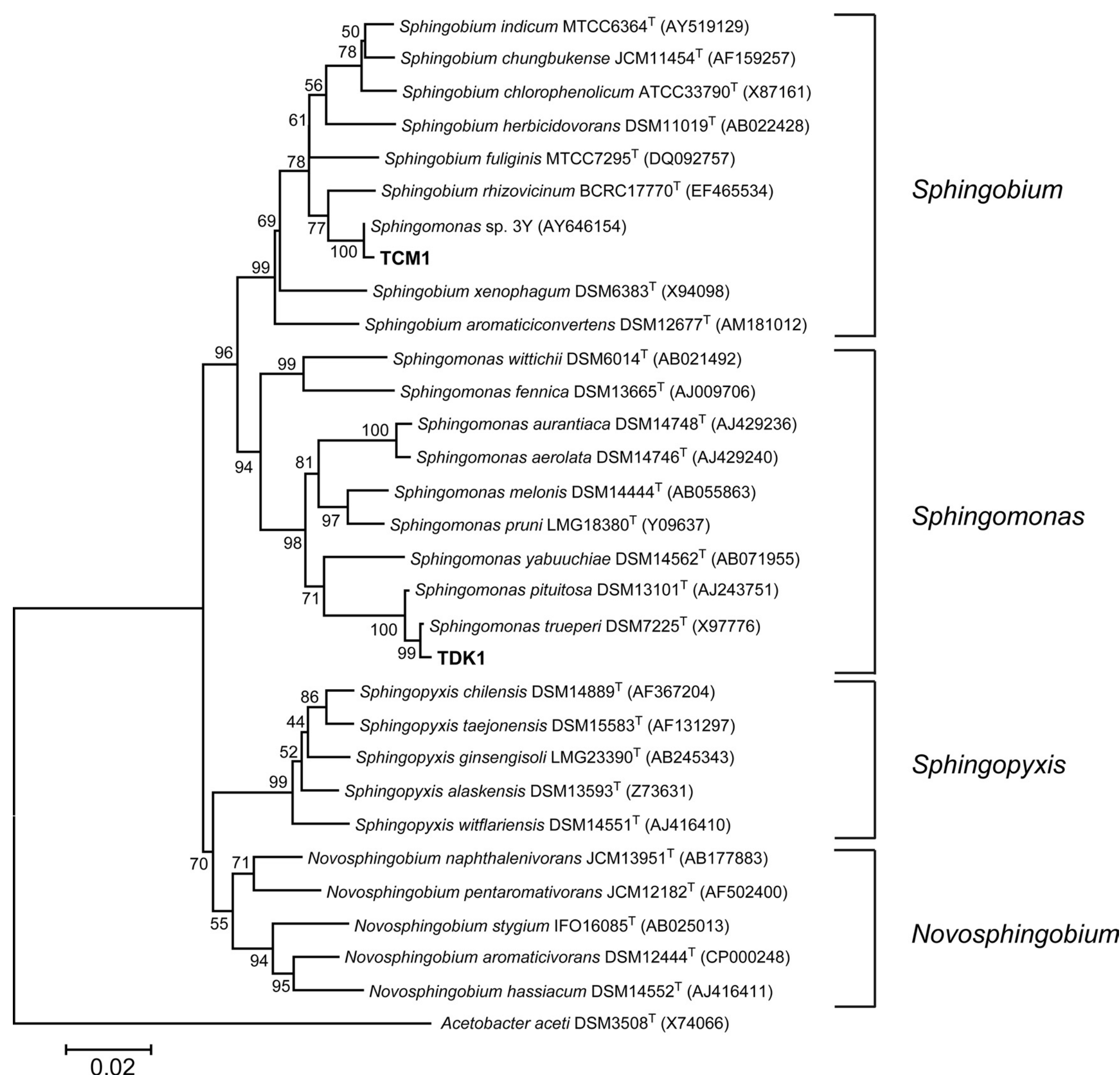

FIG. 1. Phylogenetic affiliation of isolated strains TCM1 and TDK1 based on the 16S rRNA gene sequence data. The lengths of 16S rRNA genes of strains TCM1 and TDK1 were 1,446 and 1,444 bp, respectively, without primer-binding sites. GenBank accession numbers are provided in parentheses. The dendrogram was generated by the neighbor-joining method using MEGA4 with maximum composite likelihood (http://www.megasoftware.net/). Alignment gaps and unidentified base positions were excluded from the analysis. The numbers at the branch points are the percentages of 1,000 bootstrapped data sets. The bar indicates 20 nucleotide substitutions per 1,000 nucleotide positions. *Acetobacter aceti* DSM3508^{T} (accession number X74066) was used as an outgroup.

A-Cl plate containing 232 μM TCEP (Tokyo Kasei, Japan) as the sole phosphorus source and incubated at 30°C for 3 days. Single colonies were picked into 1 ml of saline, and an aliquot (50 μl) of the cell suspension was transferred into 10 ml of A-Cl medium containing 20 μM TCEP as the sole phosphorus source and incubated at 30°C for 72 h with shaking at 165 rpm. This single-colony isolation procedure was repeated three times, and, finally, a single colony was picked and named strain TCM1. The purity of these strains was checked by microscopy using a phase-contrast microscope (BX51; Olympus Optical, Japan) and denaturing gradient gel electrophoresis (DGGE) analysis by a previously described procedure (16). The isolated strains were short-rod-shaped bacteria (0.8 to 1.0 by 1.0 to 2.5 μm) and produced yellow, circular, convex colonies with smooth, glistening surfaces on nutrient agar plates. The physiological test for identification was performed using an API Biochemical Identification Kit (Api20NE; bioMérieux, France). As a carbon source, both strains assimilated glucose, maltose and L-arabinose; in addition, TCM1 also assimilated potassium gluconate, and TDK1 assimilated D-mannose, *N*-acetyl-D-glucosamine, and DL-malate. Both strains were negative for indole, urease, arginine dihydrolase, nitrate reduction, gelatin hydrolysis, and glucose fermentation and were positive for esculin hydrolysis. TCM1 and TDK1 were negative and positive for cytochrome oxidase, respectively. The morphological and physiological characteristics of both strains were similar to those of *Sphingomonas* spp. although the two strains were distinct from each other in physiological characteristics.

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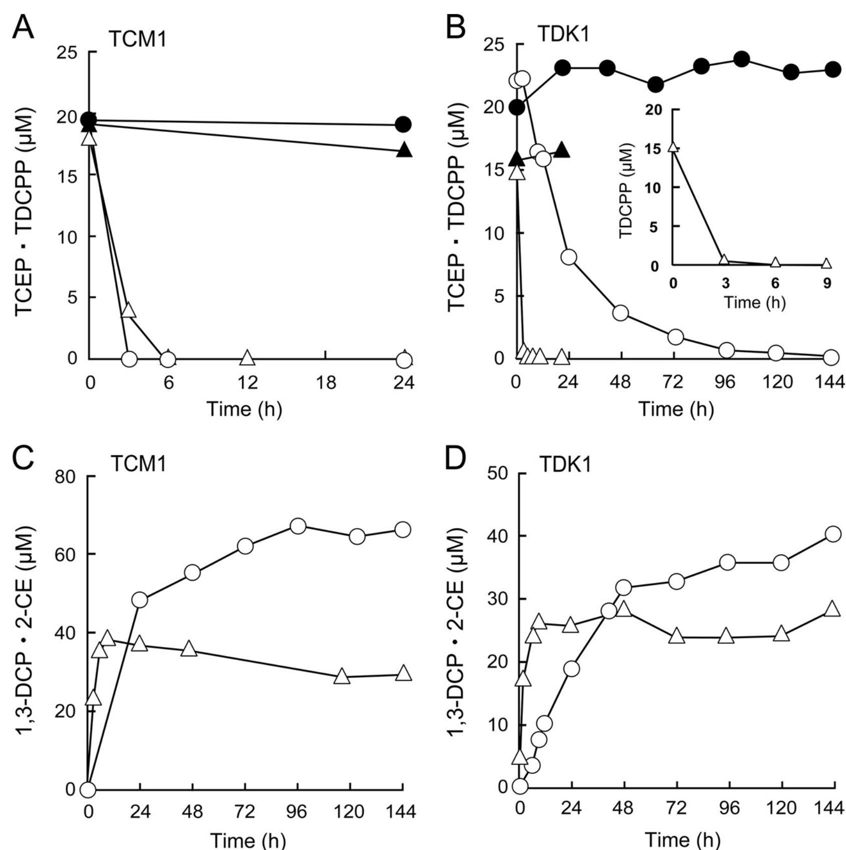

FIG. 2. Degradation of chlorinated OP flame retardants by strains TCM1 (A) and TDK1 (B) and generation of 2-CE and 1,3-DCP (C and D). The cultivations were performed aerobically at 30°C in a synthetic minimal medium containing 20 μM TCEP or TDCPP as the sole phosphorus source. (A and B) Open circles and triangles represent the concentrations of TCEP and TDCPP, respectively, and their filled forms represent concentrations for autoclaved control cells. (C and D) Open circles and triangles represent the concentrations of 2-CE and 1,3-DCP, respectively. Each data point represents the mean of at least two independent determinations and includes experimental errors of up to ±13.1, 11.4, 9.5, and 12.2% for TCEP, TDCPP, 2-CE, and 1,3-DCP, respectively.

To identify the strains using 16S rRNA gene sequences, total DNA was prepared essentially according to the procedure described by Girvan et al. (5). The nearly full-length 16S rRNA gene (approximately 1,500 bp) was amplified by PCR using the eubacterial universal primers (10PF and 1541PR) (20) and *ExTaq* DNA polymerase (Takara Bio, Japan). The amplified products were cloned into pGEM-T vector (Promega) and sequenced on both strands at least twice using an ALF Express II automated DNA sequencer (Amersham Biosciences, Sweden) and a Thermo Sequenase Cy5 Dye Terminator Kit (GE Healthcare, United Kingdom). Sequence homology searches were performed against the 16S rRNA gene sequence database at the DDBJ using the BLAST program (http://www.ddbj.nig.ac.jp/search/top-e.html). The similarity between the sequences of TCM1 and TDK1 was 93.4%. A search in genome databases showed that the sequences are closely related to those of the sphingomonads, consisting of the genera *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* (Fig. 1) (17). The highest identity and score were found to *Sphingomonas* sp. strain 3Y for TCM1 (99%; GenBank accession number AY646154) and *Sphingomonas trueperi* for TDK1 (99%; X97776). For the phylogenetic analysis of the strains, the sequences were first aligned to those of sphingomonads using Clustal X, version 2.0 (8), and a phylogenetic tree was constructed by the neighbor-joining method using MEGA4 with the maximum composite likelihood method (18). Alignment gaps and unidentified base positions were excluded from the analysis. The strains TCM1 and TDK1 were grouped with the members belonging to the genera *Sphingobium* and *Sphingomonas*, respectively, showing that strains TCM1 and TDK1 are members of the respective genera.

The ability of the strains to degrade TCEP and TDCPP was determined. Strains TCM1 and TDK1 were each precultivated in 100 ml of A-Cl medium containing 10 μM TCEP and TDCPP, respectively, at 30°C for 4 days (TCM1) and 2 days (TDK1). Cells were harvested by centrifugation at 2,500 × *g*, washed at least twice with A-Cl medium lacking a phosphorus source, and resuspended in the same fresh medium to an OD_{660} of 10. The cell suspensions were transferred to 100 ml of A-Cl medium containing 20 μM TCEP or TDCPP to give an initial OD_{660} of 0.05 and incubated at 30°C with shaking at 165 rpm. The OP compounds in cultures (0.5 ml) were extracted with equal volumes of ethyl acetate and analyzed using a GC-17A gas chromatograph (Shimadzu, Japan) as described previously (16). TCM1 completely degraded about 20 μM TCEP and TDCPP within 3 h and in 6 h, respectively (Fig. 2A). TDK1

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TABLE 1. Substrate specificity of strains TCM1 and TDK1

Substrate (group and/or compound)[a]	Growth of the indicated strain (OD_{660})[b]	
	TCM1	TDK1
None	0.041 ± 0.011	0.059 ± 0.016
NaH_2PO_4	0.891 ± 0.171	0.353 ± 0.093
Trihaloalkyl		
TCEP	0.741 ± 0.045	0.249 ± 0.084
TDCPP	0.488 ± 0.219	0.327 ± 0.047
TBPP	0.585 ± 0.072	0.348 ± 0.039
Triaryl		
TCP	0.735 ± 0.063	0.314 ± 0.047
TPP	0.699 ± 0.045	0.340 ± 0.042
Trialkyl		
TBP	0.293 ± 0.056	0.059 ± 0.019
TBXP	0.116 ± 0.013	0.058 ± 0.010
TEP	0.104 ± 0.011	0.048 ± 0.015
TEHP	0.055 ± 0.032	0.060 ± 0.013
TMP	0.102 ± 0.006	0.050 ± 0.016

[a] TCEP, tris(2-chloroethyl) phosphate; TDCPP, tris(1,3-dichloro-2-propyl) phosphate; TBPP, tris(2,3-dibromopropyl) phosphate; TCP, tricresyl phosphate; TPP, triphenyl phosphate; TBP, tributyl phosphate; TBXP, tris(2-butoxyethyl) phosphate; TEP, triethyl phosphate; TEHP, tris(2-ethylhexyl) phosphate; TMP, trimethyl phosphate.

[b] Growth was measured after 96 h. Data represent mean ± standard deviation of three independent experiments. The initial OD_{660} of cultures was 0.05.

completely degraded TDCPP in 6 h but required a much longer time of 144 h for TCEP (Fig. 2B). These results showed that both strains can degrade both OP flame retardants although TDK1 has much less degrading ability than TCM1 for TCEP.

The generation of a possible metabolite, 2-chloroethanol (2-CE) and 1,3-dichloro-2-propanol (1,3-DCP) from TCEP and TDCPP, respectively, in the cultures was determined. The metabolites in culture (0.5 ml) were extracted twice with equal volumes of ethyl acetate and identified and quantified by comparison with their respective authentic standards (Sigma-Aldrich) using a GCMS-QP2010 gas chromatograph mass spectrometer (Shimadzu) as described previously (16), except that an Econo-Cap Carbowax (EC-wax) capillary column (Alltech) was used for 1,3-DCP. 1,3-DCP was found in the culture of both strains with TDCPP, and after 9 h the concentrations reached roughly constant levels of approximately 40 μM and 26 μM in strains TCM1 and TDK1, respectively. 2-CE was found in the culture of both strains with TCEP, and the concentrations reached maximum levels of approximately 67 μM at 96 h and 40 μM at 144 h in strains TCM1 and TDK1, respectively. These results showed that the bacteria degrade the OP compounds by hydrolyzing their phosphotriester bonds. Because the strains grew using the OP compounds as the sole phosphorus sources, they can cleave all the phosphoester bonds in the compounds.

To determine whether the strains were able to degrade other OP flame retardants and plasticizers, the strains were cultivated in 100 ml of A-Cl medium containing a 5 μM concentration of various OPs as the sole phosphorus source at 30°C with shaking at 165 rpm. The cell growth was monitored at the OD_{660} after 96 h of cultivation (Table 1). No growth of the strains was observed without a phosphorus source. In addition to growth on TCEP and TDCPP, both strains were able to grow on the other trihaloalkyl phosphate, tris(2,3-dibromopropyl) phosphate (TBPP), and on triaryl phosphates, tricresyl phosphate (TCP) and triphenyl phosphate (TPP). In contrast, no growth was observed for strain TDK1 on any trialkyl phosphate tested, whereas TCM1 was able to grow moderately on tributyl phosphate (TBP) and slightly on tris(2-butoxyethyl) phosphate (TBXP), triethyl phosphate (TEP), and trimethyl phosphate (TMP). These results showed that the strains can degrade and assimilate not only the chlorinated OP flame retardants but also several other OP flame retardants and plasticizers and that the strains have different substrate specificities for trialkyl phosphates.

In summary, we have successfully isolated two bacterial strains capable of degrading persistent chlorinated OP flame retardants, TCEP and TDCPP. The two strains belong to *Sphingomonas* sp. and *Sphingobium* sp. and degraded the compounds by hydrolyzing their phosphotriester bonds, generating 2-CE and 1,3-DCP as metabolites from TCEP and TDCPP, respectively. This is the first report of isolated bacteria that can degrade persistent OP flame retardants. The bacteria may be useful for the bioremediation of sites contaminated with the persistent compounds, especially leachate and raw water of waste disposal sites, and possibly provide new insights into microbial degradation of OP compounds. Further studies to elucidate the enzymes and the genes involved in the degradation are now being undertaken.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of strains TCM1 and TDK1 have been deposited in the DDBJ, EMBL, and GenBank sequence databases under accession numbers AB331643 and AB331644, respectively.

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