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Removal of Methylmercury and Tributyltin (TBT) Using Marine Microorganisms

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Abstract Two marine species of bacteria were isolated that are capable of degrading organometallic contaminants: *Pseudomonas balearica*, which decomposes methylmercury; and *Shewanella putrefaciens*, which decomposes tributyltin. *P. balearica* decomposed 97% of methylmercury (20.0 μg/L) into inorganic mercury after 3 h, while *S. putrefaciens* decomposed 88% of tributyltin (55.3 μg Sn/L) in real wastewater after 36 h. These data indicate that the two bacteria efficiently decomposed the targeted substances and may be applied to real wastewater.

Keywords Methylmercury · TBT · *Shewanella putrefaciens* · *Pseudomonas balearica*

Organometallic compounds are found in the environment where they are naturally formed there, or they have been introduced by anthropogenic sources. Organomercury compounds were used globally for a long time as a cereal seed dressing, and in the paper pulp industry where phenylmercury acetate was used as a fungicidal additive. Besides anthropogenic sources, methylmercury and trimethyllead are also produced by algae and bacteria in aquatic systems (Rodriguez-Gonzalez et al. 2005). In the manufacturing processes, catalytic inorganic mercury compounds were unintentionally converted into methylmercury that was discharged with wastewater into nearby fishing grounds. Additionally, inorganic mercury was transformed to methylmercury through biomethylation by microorganisms and algae. Methylmercury accumulated with trophic level in the aquatic food web, and human populations that depended heavily on a fish-rich diet were exposed to considerable amounts of methylmercury (Clarkson 2002).

Tributyltin (TBT) was used extensively as a wood preservative, and as a biocide in antifouling paint applied to marine vessels. About 500 tons/year were manufactured and sold in the EU (ECHA 2009).

TBT is highly toxic to marine organisms at very low concentrations. Its use on both large and small vessels has resulted in adverse effects upon mollusks in marine harbors, such as gastropod imposex, larval mussel mortality, and oyster shell malformation (Díaz et al. 2007). This compound is very toxic to marine organisms at very low concentrations.

TBT can be transformed in aqueous media by light and microbes into the less toxic dibutyltin and monobutyltin species. These butylated tin species are quite stable in sediments, where they accumulate. TBT is also bio-accumulated by living organisms from the water–sediment interface, causing severe and long-term toxic effects to the local fauna with an impact on biodiversity and human health (Cima et al. 2003). Zabaljauregui et al. (2007) reported concentrations of methylmercury and butyltin compounds in oysters: 65–149 ng/g methylmercury ($MeHg^+$); ND-92 ng/g monobutyltin (MBT); 27–140 ng/g dibutyltin (DBT); 39–1,307 ng/g tributyltin (TBT). It is desirable to remove organomercury and organotin

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compounds from wastewater discharges due to their individual and mixed toxicities (Rouleau et al. 1995).

The most commonly used approaches to remove methylmercury and TBT from water are sorption, ion exchange, precipitation and electrochemical techniques. In general, these techniques are inefficient and generate secondary wastes that must often be disposed of as hazardous materials (Crige 1986). A promising alternative for removing these compounds is biological treatment. This does not cause secondary pollution, because the parent compounds are converted to less toxic compounds (Voulvoulis and Lester 2006). In this study, microorganisms were isolated and identified from seawater that was contaminated with methylmercury and TBT. We investigated the capabilities of these microorganisms for degrading methylmercury and TBT in raw seawater.

Materials and Methods

The microbial samples that were used in this study were collected from Incheon Harbor, South Korea. Sediments contained 2.3–25.3 pg/g methylmercury and 0.6–7.9 ng/g total mercury in 2001 (Lee and Lee 2002). The concentrations of the butyltin compounds in the seawater near this harbor in 2009 were 7.0–12.2 ng Sn/L MBT, 12.5–20.4 ng Sn/L DBT, and ND-10.0 ng Sn/L TBT (Lee et al. 2011). These pollution levels implied the potential existence of bacteria that are tolerant to these substances. Seawater samples (1 L) were collected from a depth of 20 cm in order to avoid surface contaminants. Methylmercury and TBT were added to the seawater and tolerant bacteria were identified.

A methylmercury- or TBT-minimal medium was used for enrichment and isolation. The methylmercury-minimal medium contained 20.0 μg/L of methylmercury and the TBT-minimal medium contained 5.1 μg Sn/L of TBT in the filtered (0.2 μm) seawater. The medium was sterilized in an autoclave, cooled, and pH adjusted to 7.0. The plate medium contained 2.5% agar (Difco).

A 5.0 mL seawater sample was inoculated in 100 mL of the methylmercury- or TBT-minimal medium. Enrichment cultivation was carried out at 30°C for 48 h with shaking at 100 rpm. Subsamples of the enrichment cultures were transferred into fresh MMC- or TBT-minimal medium 1% (vol/vol) and incubated for 3 d. The cultivation was repeated at least three times. After several consecutive passages, the enrichment culture was streaked onto the plate medium. The colonies that formed on the plates were removed and further purified by restreaking onto the plate medium several times.

The DNA was extracted from each sample using an Ultra Clean^{TM} Soil DNA kit (MoBio Laboratories Inc., Solana Beach, CA, USA), which involved the bead-beating and spin column purification steps. The extracted 16S rDNA genes were amplified via a polymerase chain reaction (PCR) using PCR Master (Roche Applied Science, Indianapolis, IN, USA) and a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. The primer set that was used in this study was 27f (forward primer: 5′-AGAGTTTGATCATGGCTCAF-3′) and 1492 r (reverse primer: 5′-GGATACCTTCTTACGACTT-3′) (Yoon et al. 1996). All PCR work was performed under the following amplification conditions: pre-incubation at 94°C for 5 min; 35 cycles of denaturation at 94°C, annealing at 55°C, and extension at 72°C for 45 s; a single final extension at 72°C for 10 min; incubation at 4°C until further processing.

The amplified 16S rDNA was analyzed on an ABI Prizm 377 XL sequencer (Applied Biosystems, Foster city, CA, USA). The sequences were then edited with SeqEd v1.0.3 (Applied Biosystems, Scoresby, Australia). The percentage of its similarity to the reference strains found in the GenBank database was estimated within a 322-bp section of the gene. The retrieved sequences were compared with available sequences from the BLAST program of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). The sequences were aligned with the ClustalX software (Thompson et al. 1994).

Since methylmercury contaminated water was seldomly found, we prepared it artificially at a concentration of 20 μg/L based on dental unit wastewater (Stone et al. 2003). For the TBT degradation test, wastewater was collected directly from a shipyard in Busan, Korea. The raw water was slightly acidic (pH = 5.6). The concentrations of butyltin compounds were 55.3 μg Sn/L TBT, 17.6 μg Sn/L DBT, and 1.5 μg Sn/L MBT. Degradation tests were carried out using two 5.0 L Pyrex-glass reactors with an internal diameter of 60 mm and a height of 300 mm. In each reactor, the prepared microbial sample (*P. balearica* for methylmercury and *S. putrefaciens* for TBT) was added. The microbial concentrations in the reactors were 25 mg/L for *P. balearica* and 500 mg/L for *S. putrefaciens*. All the experiments were run at 30 ± 2°C. An abiotic test of all the experiments was carried out to determine the exact biodegradability.

Extraction of organotin compounds and the analytical methods employed in this study have been described previously by Lee et al. (2011). To measure both the methylmercury and inorganic mercury concentrations in the wastewater, gas chromatography-atomic absorption spectroscopy (GC-AAS) was utilized according to methods described by Puk and Weber (1994).

The temperature was maintained at 30°C until the mercury compounds reached the AAS detector after hydrogenation. A column packed with OV-1 (53 cm, 9 mm, 2.5 g

packed weight) was used, and the column temperature was kept constant at 60°C. The helium flow rate was kept constant at 90 min/mL. A quartz T tube was used to atomize the mercury compounds and the temperature was kept constant at 835°C. A Perkin Elmer 2380 (Perkin Elmer, Norwalk, CT, USA) with a 0.2 mm slit width and a 253.7 nm wavelength was used for atomic absorption spectroscopy. An EDL system 2 (Perkin Elmer, Norwalk, CT, USA) was used as the lamp. The retention times were 2.4 min for inorganic mercury, 3.5 min for methylmercury, and 4.6 min for the internal standard of dimethylmercury. The detection limit was calculated using the regression analysis method employed by the EPA (Miller and Miller 1988). The detection limits for inorganic mercury and methylmercury were 0.3 and 0.9 μg/L, respectively.

An HP 5890 gas chromatograph equipped with a FPD (610 nm filter) was used for all butyltin determinations. A 25 m × 0.32 mm × 0.52 μm Ultra-1 (Agilent) capillary column was used with splitless injection (220°C) and the FPD was maintained at 270°C. Helium (20 psi) was used as the carrier gas in the splitless mode for 90 s. The GC temperature program was 100°C for 2 min, followed by an increase from 100 to 220°C at a rate of 15°C/min, with a final hold at 220°C for 10 min. The retention times of TET, MBT, DBT and TBT were 3.1, 4.9, 6.6 and 8.0 min, respectively. The detection limit was calculated using the regression analysis method employed by the EPA. The detection limits for MBT, DBT, and TBT were 3.4, 2.5, and 5.3 ng Sn/L, respectively.

Results and Discussion

The nucleotide sequences were determined to identify two microbes capable of degrading methylmercury or TBT in seawater. BLASTN analysis was performed to examine the similarity of the isolated clones to the reference strains in the GenBank database. All of the displayed nucleotide sequences shared >99% similarity to a reference strain. As shown in Fig. 1(a), the methylmercury-degrading microbe was closely related to *Pseudomonas* spp. (especially, *P. balearica*).

Frequently found in the marine environment, *Pseudomonas* is known to decompose toxic substances, including aromatic hydrocarbons, crude oil, and organometallic compounds (Crige, 1986). *P. balearica* belongs to the *P. stutzeri* group (Sorokin et al. 1999), and is reportedly a denitrifying microbe in marine conditions (Krieger et al. 2006). This microorganism is also thought to have the ability to oxidize thiosulfate and decompose carbon tetrachloride (Sepúlveda-Torre et al. 2002; Lalucat et al. 2006). Its capacity to degrade methylmercury, however, has not been previously reported.

Figure 1(b) shows the phylogenetic diversity of the *Shewanella* microbial community. *S. putrefaciens* is a facultative anaerobe that can metabolically reduce iron, manganese, and uranium in high salinity environments (Rossello-Mora et al. 1994; Larsen et al. 1998; Dho 2003). Ansari et al. (2007) revealed that *S. putrefaciens* could also decompose dibenzothiphene. In terms of TBT degradation, Cruze et al. (2007) reported that *S. putrefaciens* was tolerant of TBT, but had no ability to either decompose it or to grow on it. The significant finding of our study is that *S. putrefaciens* can both decompose TBT and grow on it.

As shown in Fig. 2(a), methylmercury decomposition in the abiotic test was barely observed during the 3 h reaction time. In the reactor with *P. balearica*, however, the methylmercury concentration was 10.0 μg/L after 1 h and 0.6 μg/L after 3 h, indicating that methylmercury was reduced by 94% (Fig. 2(b)).

The concentration of inorganic mercury, a byproduct of methylmercury, was 9.0 μg/L after 1 h of reaction time and

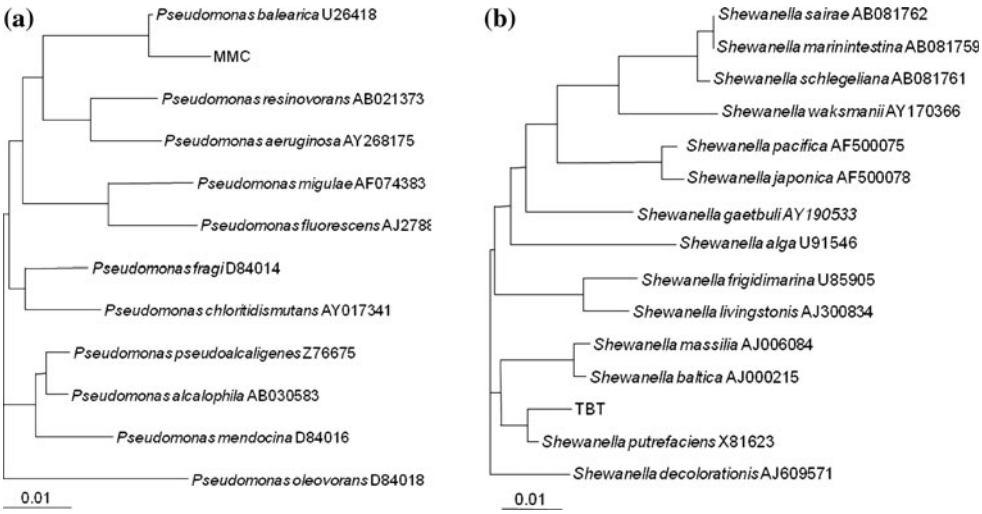

Fig. 1 Phylogenetic tree of the 16S rDNA gene of the strains (**a**) *P. balearica* and (**b**) *S. putrefaciens* (Reid and Gordon 1999; Anzai et al. 2000; NCBI 2009)

19.2 μg/L after 3 h of reaction time, indicating that the inorganic mercury increased as methylmercury was degraded. Figure 2(c), (d) shows the chromatograms at reaction times of 0 and 3 h, wherein methylmercury was nearly completely transformed.

Many microorganisms, including *E. aerogenes* and *P. fluorescens,* were previously reported to be methylmercury-decomposing strains. These two species decomposed 58%–79% of 1.0–2.5 mg/L methylmercury within 16–20 days, although quantitative comparison was significantly difficult because of the different concentrations of methylmercury and microorganisms (Mason et al. 1979; Shariat et al. 1979).

The decomposition and adsorption of TBT in the control group that had no *S. putrefaciens* were examined. The initial concentration was 55.3 μg Sn/L TBT and decreased to 7.1 μg Sn/L after 36 h. At that time, the concentrations of DBT and MBT were 7.7 and 5.96 μg Sn/L, respectively. The observation that DBT and MBT did not increase in concentration may have been attributed to their adsorption by many substances, including paint particles and microorganisms (Fig. 3(a)).

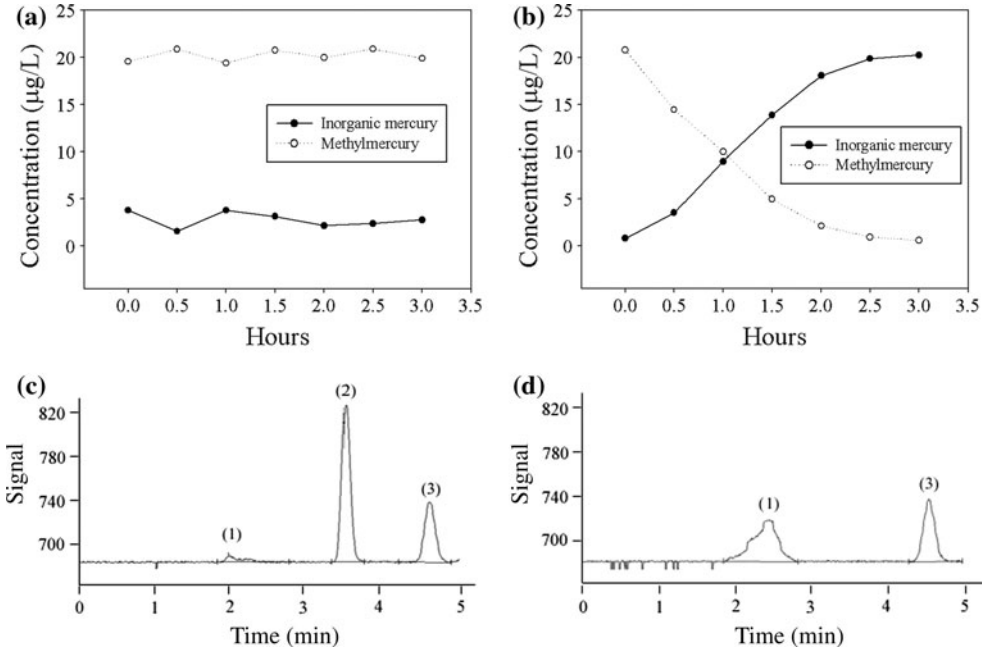

Fig. 2 Degradation of methylmercury in (**a**) the control and (**b**) the specimen with *P. balearica*, and comparison of the chromatograms in the case where *P. balearica* was used after (**c**) 0 h and (**d**) 3 h. (1) mercury ion, (2) methylmercury, (3) dimethylmercury (internal standard)

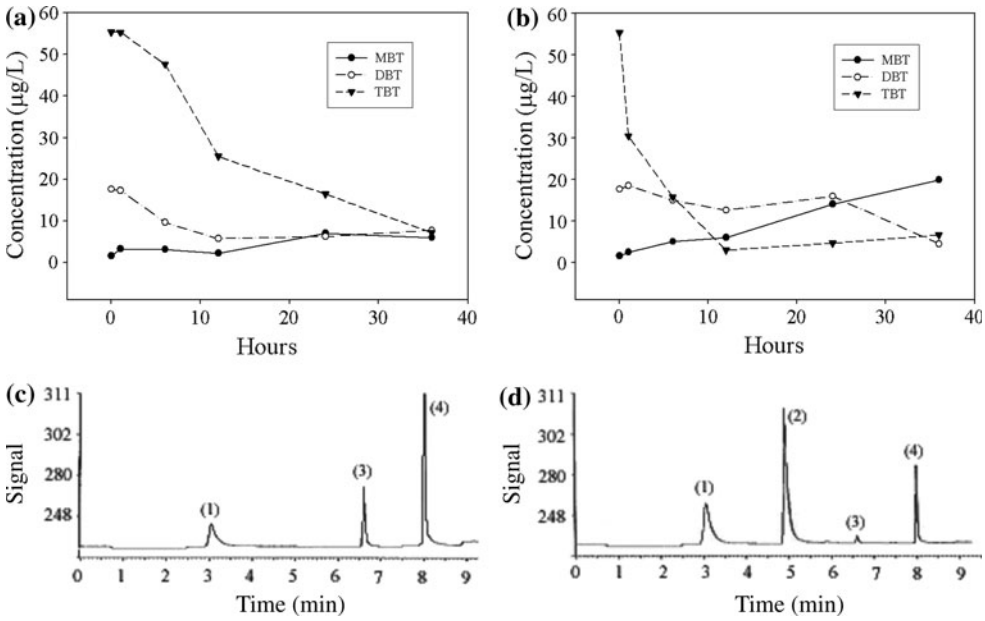

Fig. 3 Degradation of TBT in (**a**) the control and (**b**) the specimen with *S. putrefaciens*, and comparison of the chromatograms when *S. putrefaciens* was used after (**c**) 0 h and (**d**) 36 h, based on (1) TET (the internal standard), (2) MBT, (3) DBT, and (4) TBT

The results of the decomposition of TBT with *S. putrefaciens* showed that TBT decreased to 2.9 μg Sn/L after 12 h of reaction time. However, the final TBT concentration after 36 h was 6.6 μg Sn/L, implying that the incompletely biodegraded TBT had re-dissociated. The DBT concentration remained within 12.5–18.4 μg Sn/L during 24 h, but sharply decreased to 4.4 μg Sn/L after 36 h. The steady increase in MBT as both TBT and DBT decreased suggests a sequential decomposition of TBT into DBT, and of DBT into MBT. The concentration of the final MBT product gradually increased from 1.5 to 19.8 μg Sn/L (Fig. 3(b)). Considering Fig. 3(a), where the preceding decomposition was not observed, it seems that the degradation of MBT and DBT shown in Fig. 3(b) occurred due to the addition of *S. putrefaciens* to the wastewater. This decomposition pattern was similar to the TBT decomposition pattern of *P. diminuta* reported by Kawai et al. (1998). Fig. 3(c), (d) shows the chromatogram results from 0 h to 36 h, which show an increase in the MBT peak.

Other literature on microorganism-based TBT removal was examined. Active sludge removed 90% of the 100 μg Sn/L TBT in 24 h, *P. diminuta* removed 90% of the 20 μg Sn/L TBT in 24 h, and *C. vulgaris* removed 78% of the 100 μg/L TBT in 14 days, although quantitative comparison was very difficult because of the different TBT and microorganism concentrations (Stasinakis et al. 2005; Kawai et al. 1998; Tsang et al. 1999).

In summary, methylmercury- or TBT-degrading bacteria were isolated and identified in seawater. *P. balearica* decomposes methylmercury and *S. putrefaciens* decomposes TBT. These results indicate that the two bacteria can efficiently decompose toxic methylmercury and TBT and may thus find application in degrading these compounds in real wastewater.

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