Isolation and Identification of *Sphingomonas* sp. That Yields tert-Octylphenol Monoethoxylate under Aerobic Conditions

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Topsoil samples were collected from eight golf courses in Yamaguchi Prefecture, Japan, and enrichment cultures were carried out with a basal-salt medium containing 0.2% 4-tert-octylphenol polyethoxylate (OPPEO) as sole carbon source. OPPEO-degrading activity was detected in one of the samples, from which a strain of OPPEO-degrading bacterium was isolated. The isolated bacterium grew on a nutritionally enriched medium (NE medium) containing 0.2% OPPEO as sole carbon source, and accumulated 4-tert-octylphenol diethoxylate (OP2EO) (63%), 4-tert-octylphenol triethoxylate (OP3EO) (14%), and 4-tert-octylphenol monoethoxylate (OP1EO) (2%) after 7 d cultivation under aerobic conditions. The addition of clay mineral (vermiculite) to the medium accelerated the degradation of OP2EO (40%) and OP3EO (4%) to OP1EO (23%). This is the first report about bacteria that can degrade OPPEO to OP1EO under aerobic conditions. The strain was identified as *Sphingomonas macrogoltabidus*, based on the homology of a 16S rDNA sequence.

Key words: tert-octylphenol monoethoxylate; biodegradation; endocrine disrupter

Over the last decade, intense debate has focused on the effects that a number of chemicals can have an endocrine disrupting activity. The chemicals include not only many kinds of synthetic compounds such as polychlorobiphenyls (PCBs), polychlorodibenzodioxins (PCDDs), bisphenol A, p,p'-dichlorodiphenyltrichloroethane (DDT), p,p'-dichlorodiphenyldichloroethylene (DDE), organotins, and others, but also such natural products as coumestrol, genistein, daizein, and zearalenone. Alkylphenol (AP; I) and alkylphenol oligoethoxylate (APnEO; II; \(n = 1-3\)) are recognized as other kinds of important endocrine disrupters since they exhibit an estrogenic effect on aquatic organisms and some animals.2–3 APnEO (II) and corresponding carboxylate (APnEC; III; \(n = 1-3\)) (Fig. 1) have been universally detected in surface water and sediment samples.4,5 It is well known that the origins of these chemicals are nonionic surfactants alkylphenol polyethoxylate (APPEO) that are widely used as adhesives and in vehicle cleaning, household laundry, and cosmetics. They also used as a wetting and dispersing agent in pesticide formulation to improve the spreading property of the products.

There are many reports referring to the isolation and identification of degrading bacteria which can degrade APPEO, alkylphenol polyethoxylate and AP, alkylphenol; OPPEO, 4-tert-octylphenol polyethoxylate; OP3EO, 4-tert-octylphenol triethoxylate; OP2EO, 4-tert-octylphenol diethoxylate; OP1EO, 4-tert-octylphenol monoethoxylate; RFLP, restriction fragment length polymorphism.

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Abbreviations: APPEO, alkylphenol polyethoxylate; AP, alkylphenol; OPPEO, 4-tert-octylphenol polyethoxylate; OP3EO, 4-tert-octylphenol triethoxylate; OP2EO, 4-tert-octylphenol diethoxylate; OP1EO, 4-tert-octylphenol monoethoxylate; RFLP, restriction fragment length polymorphism.
the polyethylene glycol side chain of APPEO. All the isolated bacteria accumulate alkylphenol triethoxylate (AP3EO) and alkylphenol diethoxylate (AP2EO) in the culture medium,\textsuperscript{6,9} but the microorganisms that convert AP2EO to AP are not yet known under aerobic conditions. Ginkel has suggested that the complete mineralization of the surfactants is accomplished by mixed cultures of microorganisms.\textsuperscript{10}

In this study, we tried to detect APPEO degrading bacteria in the topsoil of golf courses. The golf course would appear to be a place under continuous selection pressure by APPEO since APPEO-containing pesticides are sprayed to maintain the grass condition and to control the grow of weeds.

We collected the topsoil samples from eight golf courses in Yamaguchi Prefecture, Japan, and investigated to find OPPEO degrading microorganisms. We isolated a bacterial strain that can degrade OPPEO to OP1EO. The bacterium was identified as Sphingomonas macrogoltabidus, based on 16S rDNA sequence similarity. Sphingomonas species are known for their degradation activities of many synthetic and aromatic chemicals.\textsuperscript{11–14} This is the first report of bacteria that can degrade OPPEO to OP1EO under aerobic conditions.

Materials and Methods

Materials and reagents. The OPPEO surfactant (Triton X-100) was purchased from Aldrich Chemicals (Milwaukee, U.S.A.). This compound contains a branched chain octylphenol attached to polydisperse polyethylene glycol units averaging n = 9.5 (Fig. 1).

All other reagents, including high-performance liquid chromatographic grade acetonitrile, methanol, and ethyl acetate were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Apparatus. GCMS analyses were performed with a Perkin Elmer GCMS Q-910 instrument equipped with a Supelco SPB\textsuperscript{TM} column (30 m length, 0.32 mm diameter) automatic injector.

HPLC analyses were performed with a Shimadzu LC-6B instrument equipped with a Shodex MS pak GF-310 4E column (4.6 × 250 mm). The detection wavelength was 223 nm. Samples were injected via a 5 μl injection loop and eluted isocratically in acetonitrile–water (3:7 [vol/vol]) at a flow rate of 0.3 ml/min.

PCR amplification was performed with a TP-500 DNA thermal cycler (Takara Biochemicals, Shiga, Japan).

Soil samples. The soil samples were collected in February 2002 from eight golf courses in Yamaguchi Prefecture, Japan.

Culture media. Basal-salt medium was prepared in sterile water. The basal salts contained 0.2% OPPEO (wt/vol) as the sole added carbon source. They contained (grams/liter) Na\textsubscript{2}HPO\textsubscript{4} (6.8), KH\textsubscript{2}PO\textsubscript{4} (3.0), NaCl (0.5), (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} (2.0), MgSO\textsubscript{4} (0.24), CaCl\textsubscript{2} (0.01), and OPPEO (2.0).

The NE medium (ml/100 ml) was prepared from the basal salts (99.6), a vitamin solution (0.2), a trace element solution (0.1), A5 (0.1), and 0.05% yeast extract. The vitamin solution and trace element solution were prepared as reported previously.\textsuperscript{9} The A5 solution (mg/100 ml) contained H\textsubscript{2}BO\textsubscript{3} (286.0), MnSO\textsubscript{4}·7H\textsubscript{2}O (250), ZnSO\textsubscript{4}·7H\textsubscript{2}O (22.2), CuSO\textsubscript{4}·5H\textsubscript{2}O (7.9), and Na\textsubscript{2}MoO\textsubscript{4}·2H\textsubscript{2}O (2.1). The 1/2 LB medium (grams/100 ml) contained bacto-tryptone (0.5), bacto-yeast extract (0.25), and NaCl (0.25).

Isolation and maintenance. Each soil sample (5 g) was inoculated into the sterile basal-salt medium containing 0.2% (wt/vol) OPPEO as the only carbon source (20 ml, pH 7.5). The enrichment culture was carried out at 30\textdegree C and 120 rpm agitation. The supernatant of the culture medium was transferred to new NE medium at an interval of 1 week. After several transfers, the bacteria were separated by the streak method. The colonies appearing on the NE agar medium were collected and preserved on the agar slants with the same medium constitution. The isolated OPPEO-degrading bacterium was designated G-3.

Analysis of OPPEO biodegradation by the isolated bacteria. The isolated bacterium G-3 was preliminarily cultured on an agar slant NE medium containing 0.2% Triton X-100. The bacterium was suspended in the NE medium to make up a bacterial suspension (OD = 1 at 660 nm). This suspension (500 μl) was inoculated into fresh NE medium (20 ml), and the biodegradation test was carried out at 120 rpm and 30\textdegree C. After 7 d of incubation, the culture medium (500 μl) was extracted with an equal volume of ethyl acetate. The ethyl acetate layer (1 μl) was analyzed by GCMS.

Determination of degradation product in the presence of vermiculite. The bacterial suspension (OD = 1 at 660 nm, 500 μl) was inoculated into fresh NE medium (20 ml) and the same medium (20 ml) containing sterilized vermiculite (2 g). The media were agitated at 120 rpm and 30\textdegree C. After 7 d of incubation, each of the culture media (500 μl) was extracted with an equal volume of ethyl acetate and the layer was analyzed by GCMS. The ethyl acetate layer (100 μl) was evaporated in vacuo and methanol (350 μl) was added. The methanol layer was analyzed by HPLC.

The contents of OP3EO, OP2EO, and OP1EO in the culture medium were determined by their peak areas by HPLC chromatogram. The conversion ratios were indicated as molar fractions of the degradation products to the original Triton X-100.
MALDI-TOF MS analysis of culture media. The MALDI-MS measurements using a Voyager DE-PRO time-of-flight mass spectrometer (Applied Biosystems, Tokyo, Japan) equipped with a pulsed nitrogen laser and a delayed extraction ion source were described previously. In brief, laser beam intensity was experimentally attenuated to just above the threshold for analyte ionization. Ions generated by the laser desorption were introduced into the flight tube with an acceleration voltage of 20 kV in the high-resolution reflector (2.0 m flight path) positive ion mode. The delay time setting was typically 50 ns.

As the matrix for sample ionization, 5, 10, 15, 20-tetrakis (pentafluorophenyl)-porphyrin (F20TPP, Sigma Chemical, St. Louis, U.S.A., MW = 974.6) was used, and about 2 mg of F20TPP was dissolved in 1 ml of ethyl acetate to make the matrix solution. Prior to the sample/matrix co-crystal deposition, 1 ml of a 1 mmol sodium iodide solution in acetone was spotted onto a flat stainless-steel sample plate and dried in air to deposit fine NaI crystals as a cationization salt. Then 1 ml of the sample/matrix solution was pipetted onto the thin NaI crystal layer, and dried in air. All mass spectra were collected by averaging 500 individual laser shots.

Genomic DNA preparations of degrading bacteria. Genomic DNA of isolated bacterium was extracted as described by Saito and Muira. The purity of the extracted DNA was checked by the 230:260 and 280:260 nm absorbance ratios.

PCR amplification of 16S rDNA fragment. PCR amplification was carried out with a 50 µl volume consisting of 33.7 µl of sterile distilled water, 5 µl of 10 × PCR buffer, 4 µl of each deoxyribonucleotide triphosphate (2.5 mmol), 1 µl of each primer (50 pmol), 1.3 µl of Taq DNA polymerase (1 U/µl, Roche, Basel, Switzerland), and 4 µl of template DNA (0.1 µg). Amplification was performed in an automated DNA thermal cycler under the following conditions: denaturation at 94 °C for 2 min and 30 cycles of denaturation at 94 °C for 30 sec, primer annealing at 55 °C for 60 sec, and primer extension at 72 °C for 60 sec. The primers were designed from the conserved region of the 16S rDNA sequence of *E. coli*. The forward primer was 5’GCTCAGATTTGAACGGCTGCG3’ (41f), corresponding to the 22–41 positions, and reverse primer was 3’GTCGAGCACCAACTTTACAS’ (1,066r), corresponding to the 1,066–1,085 positions.

16S rDNA RFLP. Amplified 16S rDNA was digested by selected restriction enzymes, Hae III, Hha I, and Alu I. A similarity search was made by using the program of Watanabe and Okuda. This program provides 1,533 kinds of 16S rDNA RFLP database consisting of 378 kinds of bacterial genus and 1,288 bacterial species.

Sequencing of DNA. The sequence of the amplified 16S rDNA was determined at the Shimadzu Genomic Research Center. The whole sequence of amplified fragments of approximately 1,000 bp was determined. The similarity of the 16S rDNA sequences between strain G-3 and other species was compared with all known sequence data in the GenBank, DDBJ databases using the BLAST algorithm. Phylogenetic trees based on the 16S rDNA sequences were constructed using the Clustal X program of the neighbor-joining method.

Results and Discussion

Isolation and identification of the OPPEO-degrading bacteria

Each soil sample collected was shaken with the basal-salt medium for 1 week, and the resulting suspension was extracted with an equal volume of ethyl acetate. The organic layer was analyzed by HPLC. One soil sample exhibited OPPEO degrading activity. The HPLC chromatogram for this extract is shown in Fig. 2. The biodegradation products were identified as OP2EO and OP1EO by comparing their retention times with those of corresponding authentic samples. A bacterial strain was isolated from the soil sample with OPPEO-degrading activity. The new strain, designated G-3, was gram negative and aerobic, and was able to degrade OPPEO in pH 7.5 but unable to degrade it in pH 5 or pH 9. The color of the colony of the strain was pale yellow and yellow on the NE medium and the 1/2 LB medium respectively.

Genomic DNA of the isolate G-3 was extracted and PCR-RFLP analysis derived from 16S rDNA was carried out according to the method of Watanabe and Okuda. The analysis showed a low level match to *Asticcacaulis excentricus*, *Caulobacter subvibrioides CB81*, *Sphingomonas mali*, and *Sphingomonas asac-

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**OP2EO 98.9 min**

**OP1EO 139.7 min**

**Fig. 2.** HPLC Chromatogram of the Biodegradation Products of OPPEO When Incubated with the Topsoil of a Golf Course. Biodegradation products after 1 week of incubation in basal-salt medium. Column: Shodex Mspak GF-310E (4.6 × 250 mm); column temperature: 40 °C; solvent: 30% acetonitrile; flow rate: 0.3 ml/min; detection wavelength: 223 nm. The incubation medium (500 µl) was extracted with the equal volume of ethyl acetate. The ethyl acetate layer was injected via a 5 µl injection loop.
Then the base sequence of the PCR product was identified. The determined sequence of the fragment was about 1,000 bp long. The sequences were also determined and aligned. Sequence analysis clearly showed that strain G-3 was most similar to *Sphingomonas macrogoltabidus*. It indicated that the G-3 strain had high sequence homology (98% identity) to *S. macrogoltabidus*, as shown in Fig. 3.

*Sphingomonas* species are known for their degradation of many synthetic compounds. For example, Hernaez *et al.* showed that a strain identified as *Sphingomonas macrogoltabidus* is a tetralin-utilizing bacterium that can grow on tetralin as sole carbon source in the wide range of pH 5.3–9.11 *Sphingomonas cloacae* degraded nonylphenol,12 and *Sphingomonas aromaticivorans*13 and *Sphingomonas* sp.14 degraded several polycyclic aromatic hydrocarbons.

**GCMS analysis of the biodegradation products of isolated bacteria G-3**

To determine the degradation products of OPPEO by the bacterial isolate G-3, the products were analyzed by GCMS after 7 d of incubation. The total ion chromatogram showed two peaks with retention times of 10.41 min (A) 8.93 min (B) (Fig. 4). The spectra for (A) and (B) gave the M+ ions at 294 and 250, corresponding to OP2EO and OP1EO respectively. They gave characteristic fragment ions at \( m/z = 223 \) and \( 135 \), and \( m/z = 179 \) and 135, corresponding to OP2EO and OP1EO respectively. These derivatives had identical retention times and MS fragmentation patterns to those of the authentic samples. While some kinds of bacteria degrading APPEO to AP2EO have been reported,6–8 the microorganisms that convert AP2EO to AP are not yet known under aerobic conditions. Recent studies in our laboratory have shown that 11 *Pseudomonas* strains from different paddy fields yield OP2EO as an end product.9 John and White suggested that the degradation from AP2EO to AP is done by other organisms or by abiotic processes.7
Determination of degradation products in the presence of vermiculite

The *Sphingomonas* sp. G-3 isolated from the topsoil of a golf course is the first bacterium that can degrade OPPEO to OP1EO under aerobic conditions. Figure 5B shows that the conversion ratio from OPPEO to OP1EO by liquid culture was only 2% after 1 week of incubation. Since the conversion ratio from OPPEO to OP1EO in this experiment was very low compared to that of the previous experiment with soil and basal-salt medium (Fig. 2), we estimated the effect of the addition of clay minerals to the culture medium. We used vermiculite as the clay mineral since it contains few organic materials and is easily available. The addition of vermiculite had a positive effect on the conversion ratio to OP1EO, as shown in Fig. 5(A). The conversion ratios are indicated as molar fractions of degradation products to the original Triton X-100. The conversion ratios into OP3EO, OP2EO, and OP1EO were 4%, 40%, and 23% respectively when vermiculite was added to the medium. The conversion ratios into OP3EO, OP2EO, and OP1EO, however, were 14%, 63%, and 2% respectively when vermiculite was not added. It is obvious that the addition of clay mineral (vermiculite) promoted the subsequent degradation from OP2EO. GCMS showed that each degradation stopped at the OP1EO stage through unknown mechanisms. In our preliminary experiment, when a sterile soil was added, a similar effect was observed (data not shown).

One peak was detected at about 10.0 min in the NE medium with or without vermiculite. This peak can be predicted to be 4-tert-octylphenol oligoethoxycarboxylate (OPnEC: \( n = 2-5 \)) by comparing it with the authentic samples. The Shodex MS pak GF-310 4E column used in this experiment cannot separate these oligomers. To identify the existence of OPnEC in the culture medium, MALDI-TOF analyses were performed. The MS spectrum of the culture medium of G-3 is shown in Fig. 6. According to our previous reports,15,16) the series of ion peaks labeled with circles (●) can be attributed to OPnEO molecules with a sodium cation attached \[ R\text{-}(CH_2\text{CH}_2\text{O})_n\text{H} + \text{Na}^+; \ m/z = 44n + 206 + 23 \]. The other series (▼ and ⬤) can be assigned to the ions of carboxylated OPnEO molecules (OPnEC) with a sodium cation attached \[ R\text{-}(CH_2\text{CH}_2\text{O})_n\text{CH}_2\text{COOH} + \text{Na}^+; \ m/z = 44(n - 1) + 264 + 23 \], and to their sodium salts (OPnECNa) \[ R\text{-}(CH_2\text{CH}_2\text{O})_{n-1}\text{CH}_2\text{COONa} + \text{Na}^+; \ m/z = 44(n - 1) + 286 + 23 \] respectively. The formation of carboxylate salt ions such as OPnECNa, which might be generated mainly during sample preparation and/or ionization, is a common phenomenon in MALDI-MS measurements using sodium cationization salts. The alkylphenol ethoxycarboxylate (APnEC) are the well-known intermediates in the biodegradation of APPEO.10) Sugimoto et al. reported that *Sphingomonas* spp. utilized polyethylene glycols (PEGs) as sole carbon and energy source through oxidative PEG degradation initiated by dehydrogenase that oxidizes the terminal alcohol group of the polymer chain.20) The isolated *Sphingomonas* utilized OPPEO by oxidation of the terminal alcohol group and resulted in such short ethoxy chain homologs as OPnEC (\( n = 2-5 \)).

Our preceding studies also show that each *Pseudo-*
monas strain S1 to S11 degraded OPPEO to OP2EO but not to OP1EO when incubated in an isolation medium containing 0.2% Triton X-100 with or without vermiculite for up to 2 months.\(^9\) It is interesting that Sphingomonas sp. changed its biodegradation characteristics in the presence of the clay mineral. Vermiculite is known to have a porous surface. When Triton X-100 is dissolved in a water system, OP2EO tends to adsorb to the surface of the vermiculite.\(^8\) Therefore, contaminants like OP2EO attached to the soil surface might be degraded by soil-phase bacteria (i.e., bacteria attached to the soil grains), thereby increasing over-degradation.

References