Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

Isolation of Bacterial Strains that Produce the Endocrine Disruptor, Octylphenol Diethoxylates, in Paddy Fields

Eriko NISHIO[a], Yayoi ICHIKI[a], Hiroto TAMURA[b], Shiro MORITA[a], Katuji WATANABE[c] & Hiromichi YOSHIKAWA[a]

[a] Department of Environmental Chemistry, Kyushu Kyoritsu University Kitakyushu 807-8585, Japan

[b] Department of Applied Biochemistry, Meijyo University Nagoya 468-8502, Japan

[c] Department of Agro-Environment Resource Research, Laboratory of Soil Microbiology, National Agricultural Research Center for Kyushu Okinawa Region Kikuchi-gun, Kumamoto 861-1192, Japan

Published online: 22 May 2014.

To cite this article: Eriko NISHIO, Yayoi ICHIKI, Hiroto TAMURA, Shiro MORITA, Katuji WATANABE & Hiromichi YOSHIKAWA (2002) Isolation of Bacterial Strains that Produce the Endocrine Disruptor, Octylphenol Diethoxylates, in Paddy Fields, Bioscience, Biotechnology, and Biochemistry, 66:9, 1792-1798, DOI: 10.1271/bbb.66.1792

To link to this article: http://dx.doi.org/10.1271/bbb.66.1792

Biosci. Biotechnol. Biochem., **66** (9), 1792–1798, 2002

Isolation of Bacterial Strains that Produce the Endocrine Disruptor, Octylphenol Diethoxylates, in Paddy Fields

Eriko NISHIO,[1] Yayoi ICHIKI,[1] Hiroto TAMURA,[2] Shiro MORITA,[1] Katuji WATANABE,[3] and Hiromichi YOSHIKAWA[1,†]

[1]*Department of Environmental Chemistry, Kyushu Kyoritsu University, Kitakyushu 807-8585, Japan*
[2]*Department of Applied Biochemistry, Meijyo University, Nagoya 468-8502, Japan*
[3]*Department of Agro-Environment Resource Research, Laboratory of Soil Microbiology, National Agricultural Research Center for Kyushu Okinawa Region, Kikuchi-gun, Kumamoto 861-1192, Japan*

Received November 5, 2001; Accepted May 9, 2002

Topsoil samples were collected from 36 different paddy fields in West Japan. Each soil sample was incubated with a basal salt-medium containing 0.2% OPPEO. Twelve samples possessed OPPEO-degrading activity, from which twelve cultures of OPPEO-degrading bacteria were isolated. The isolated bacteria grew on a medium containing 0.2% OPPEO as the sole carbon source, and OP2EO and OP3EO were accumulated in the medium under aerobic conditions. OP1EO and octylphenol, which have often been identified in surface water together with OP2EO, were not observed in this experiment. The bacterial isolates were gram negative and tentatively identified as *Pseudomonas putida* (10 isolates) and *Burkholderia cepacia* (one isolate) by BIOLOG and 16S rDNA RFLP analyses.

Key words: octylphenol polyethoxylate; octylphenol diethoxylate; biodegradation; endocrine disruptor; paddy field

During the last decade, about 40 non-steroidal endocrine disruptors have been identified.[1–4] Endocrine disrupters include such degradation products of alkylphenol polyethoxylates (APPEOs) as nonylphenol polyethoxylates (NPPEOs) and octylphenol polyethoxylates (OPPEOs) that are commercially important surfactants. APPEOs are composed of a phenol nucleus that is *o*, *m*, and *p*-substituted with a hydrophobic and branched alkyl moiety, and with a hydrophilic polyethylene glycol chain linked to the phenolic oxygen. While the longer types of APnEO ($n > 3$) lack estrogenic activity, it is known that the shorter types ($n \leqq 3$) and original octylphenol and nonylphenol exhibit an estrogenic effect on aquatic organisms and on mammals and birds.[5–7] Biodegradation of APPEO has been studied, and it has already been confirmed that the primary degradation of APPEO is efficient.[8] Such degradation products as short (typically 1–3) ethoxy chain length APnEOs, APs themselves, and carboxylated APnECs, however, occur frequently as stable intermediates in effluent, activated sludge and surface water.[9–13] This means that the relatively benign surfactant is converted to more endocrine-disruptive compounds by the biodegradation process.

The less common but important emulsifier, octylphenol polyethoxylates (OPPEO), is widely used as a wetting and dispersing agent in pesticide formulations (Fig. 1). Since such metabolites as octylphenol diethoxylate, octylphenol monoethoxylate and octylphenol have been identified in surface water, some microorganisms which can degrade OPPEO seem to exist in the environment. Farm land would seem to be a place under selective pressure by the surfactant since it is spread as an adjuvant component with agricultural chemicals. The objective of this study is to find OPPEO-degrading microorganisms in paddy soil and to identify these species of the degrading microorganisms.

Materials and Methods

Materials and reagents. The OPPEO surfactant (Triton X 100) was purchased from Aldrich Chemicals (Milwaukee, U.S.A.). This compounds contains a branched chain octylphenol attached to poly-

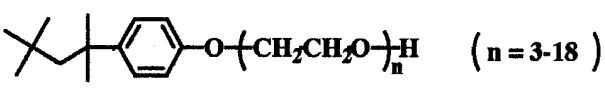

Fig. 1. Structure of OPPEO in Which n May Range from 3 to 16.
A commercial mixture also contains about 3% of polyethylene glycol.

† To whom correspondence should be addressed. Tel: +81-93-693-3167; Fax: +81-93-693-3201; E-mail: fortuna@kyukyo-u.ac.jp

Abbreviations: OPPEO, 4-(1,1,3,3-tetramethylbutyl)phenol polyethoxylate (octylphenol polyethoxylate); OP3EO, octylphenol triethoxylate; OP2EO, octylphenol diethoxylate; OP1EO, octylphenol monoethoxylate; RFLP, restriction fragment length polymorphism

disperse polyethylene glycol units averaging n = 10.

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

Preparation of the standard degradation products. a) 2-[*p*-(1′,1′,3′,3′-tetramethylbutyl)phenoxy]ethanol (OP1EO): A solution of sodium octylphenoxide (22.8 g) and ethyl bromoacetate (17.0 g) in dry DMSO (80 ml) was stirred overnight at 25°C. After the reaction had been completed, 200 ml of water was added to the reaction mixture, and the mixture was extracted with ether (200 ml × 3). The ethereal layer was washed twice with water and dried over anhydrous sodium sulfate. The ethereal layer was evaporated *in vacuo* to give a crude product. This product was purified by silica gel column chromatography with *n*-hexane-ethyl acetate (15:1) as the eluent to afford ethyl *p*- (1′,1′,3′,3′-tetramethylbutyl)phenoxyacetate as a pale yellow liquid (22.8 g; 78.1% yield). A solution of this ethyl *p*-(1′,1′,3′,3′-tetramethylbutyl)phenoxy-acetate (22.4 g/100 ml of dry THF) was added dropwise to a suspension of $LiAlH_4$ (1.83 g/50 ml of dry THF) below 5°C while stirring. After this addition, the mixture was stirred at room temperature for 3 hrs. After the reaction had been completed, ethyl acetate (20 ml) was added dropwise to the reaction mixture at below 10°C to decompose excess $LiAlH_4$. After the usual workup, crude 2-[*p*-(1′,1′,3′,3′-tetramethylbutyl)phenoxy]ethanol (OP1EO) was obtained as a pale yellow liquid. The product was purified by silica gel column chromatography with *n*-hexane-ethyl acetate (20:1) as the eluent to afford pure 2-[*p*-(1′,1′,3′,3′-tetramethylbutyl)phenoxy]-ethanol (OP1EO, 16.7 g, 87.2% yield). ^{1}H-NMR δ ($CDCl_3$): 0.71 ($CH_3 \times 3$, s), 1.34 ($CH_3 \times 2$, s), 1.70 (CH_2, s), 3.80–4.22($CH_2 \times 2$, m), 6.84 (2H, d, J = 9.0 Hz), 7.28 (2H, d, J = 9.0 Hz). ^{13}C-NMR δ ($CDCl_3$): 31.6(3C), 31.7(2C), 32.2, 37.7, 56.8, 60.0, 69.0, 113.7(2C), 127.0(2C), 142.5, 156.2. UV λmax (EtOH) nm (ε): 283 (1,350), 277 (1,580), 225 (11,080). GCMS: t_R = 9.04 min; m/z (%): 250 (M^+, 4), 179 (100), 135 (39).

b) 6-[*p*-(1′,1′,3′,3′-Tetramethylbutyl)phenyl]3,6-dioxahexanol (OP2EO): A solution of OP1EO (10 g/30 ml of dry THF) was added dropwise to a suspension of sodium hydride (60% in oil: 2.3 g/50 ml of dry THF) while stirring at room temperature. The evolution of hydrogen ceased after 1 hr of stirring, and an ethyl bromoacetate solution (7.0 g/30 ml of dry THF) was then added dropwise to the reaction mixture at 10°C. The reaction mixture was stirred at room temperature for 1 hr and then refluxed for 3 hrs. After the usual workup, crude ethyl 6-[*p*-(1′,1′,3′,3′-tetramethylbutyl)phenyl]-3,6-dioxahexanoate was obtained. The crude product was purified by silica gel column chromatography with *n*-hexane-ethyl acetate (20:1) as the eluent to afford pure ethyl 6-[*p*-(1′,1′,3′,3′-tetramethyl-butyl)phenyl]-3,6-dioxahexanoate in an 84.4% yield (11.3 g). A solution of ethyl 6-[*p*-(1′,1′,3′,3′-tetramethylbutyl)phenyl]-3,6-dioxahexanoate (11.0 g/30 ml of dry THF) was added to a suspension of $LiAlH_4$ (1.5 g/50 ml of dry THF) at below 5°C while stirring. After this addition, the mixture was stirred at room temperature for 4 hrs, before ethyl acetate (20 ml) was added dropwise to the reaction mixture at below 10°C to decompose excess $LiAlH_4$. After the usual workup, crude 6-[*p*-(1′,1′,3′,3′-tetramethylbutyl)phenyl]-3,6-dioxahexanol (OP2EO) was obtained as a pale yellow liquid. This product was purified by silica gel column chromatography with *n*-hexane-ethyl acetate (15:1) as an the eluent to afford pure 6-[*p*-(1′,1′,3′,3′-tetramethylbutyl)phenyl]-3,6-diox-ahexanol (OP2EO) in a 78.3% yield (7.5 g). ^{1}H-NMR δ ($CDCl_3$): 0.71 ($CH_3 \times 3$, s), 1.34 ($CH_3 \times 2$, s), 1.70 (CH_2, s), 3.80–4.21($CH_2 \times 4$, m), 6.83 (2H, d, J = 9.0 Hz), 7.29 (2H, d, J = 9.0 Hz). ^{13}C-NMR δ ($CDCl_3$): 31.6 (3C), 31.7 (2C), 32.2, 37.9, 56.9, 61.7, 67.2, 69.7, 72.5, 113.7(2C), 127.0(2C), 142.5, 156.2. UV λmax (EtOH) nm (ε): 283 (1,145), 277 (1,381), 225 (9,457). GCMS: t_R = 10.53 min; m/z (%): 294 (M^+, 8), 223 (100), 135 (47).

c) 9-[*p*-(1′,1′,3′,3′-Tetramethylbutyl)phenyl]-3,6,9-trioxanonanol (OP3EO): OP3EO was synthesized from OP2EO by a method similar to that for OP2EO in an 82.3% yield (5.1 g). ^{1}H-NMR δ ($CDCl_3$): 0.71 ($CH_3 \times 3$, s), 1.34 ($CH_3 \times 2$, s), 1.70 (CH_2, s), 3.61–4.25 ($CH_2 \times 6$, m), 6.83 (2H, d, J = 9.0 Hz), 7.29 (2H, d, J = 9.0 Hz). ^{13}C-NMR δ ($CDCl_3$): 31.6 (3C), 31.7 (2C), 32.2, 37.9, 56.9, 61.7, 67.2, 69.8, 70.3, 70.7, 72.5, 113.7 (2C), 126.9 (2C), 142.4, 156.2. UV λmax (EtOH) nm (ε): 283 (1,038), 277 (1,210), 225 (8,324). GCMS: t_R = 10.53 min; m/z (%): 338 (M^+, 6), 267 (100), 135 (25)

Instrumental analyses. ^{13}C- and ^{1}H-NMR spectra were recorded by a JEOL AL400 instrument, using tetramethylsilane as an internal standard.

The degradation process was tracked by an IATROSCAN TH-10 instrument (Iatron Laboratories) with Chromarod-S III.

GCMS analyses were performed by a Perkin Elmer GCMS Q-910 instrument equipped with a Supelco SPB™1 column (30 m length, 0.32 mm diameter) automatic injector.

HPLC analyses were performed by a Shimadzu LC-6A instrument equipped with a Shodex MS pak GF-310 4E column (4.6 × 250 mm).

PCR amplification was carried out with a TP-500 DNA thermal cycler (Takara Biochemicals).

Soil samples. The soil samples were collected in April 2000 from 35 different paddy fields in West

Japan (Nara prefecture, 6 locations, Osaka prefecture 5 locations, Okayama prefecture, 6 locations, Yamaguchi prefecture, 8 locations, Fukuoka prefecture, 10 locations). The water content of the each soil sample was between 18% and 32%.

Culture media. The basal-salts medium was prepared in sterile water and contained 0.2% OPPEO (wt/vol) as the sole added carbon source. The basal salts contained in this medium were (grams/liter) Na_2HPO_4 (6.8), KH_2PO_4 (3.0), NaCl (0.5), $(NH_4)_2SO_4$ (2.0), $MgSO_4$ (0.24), $CaCl_2$ (0.01), together with OPPEO (2.0). The isolation medium (ml/100 ml) was prepared from the basal salt-medium (99.7), a vitamin solution (0.2) and a trace element solution (0.1). The vitamin solution contained (mg/100 ml) pyridoxine hydrochloride (10.0), riboflavin (5.0), thiamine hydrochloride (5.0), nicotinic acid (5.0), lipoic acid (5.0), *p*-aminobenzoic acid (5.0), folic acid (2.0), biotin (2.0), and vitamin B_{12} (0.1) and the trace element solution contained (mg/100 ml) $MnCl_2 \cdot 4H_2O$ (20.0), $CoCl_2 \cdot 6H_2O$ (4.0), $Na_2MO_4 \cdot 2H_2O$ (26.0), and $FeCl_3 \cdot 6H_2O$ (15.0).

Isolation of the degrading bacteria. Each collected soil sample (5.0 g) was inoculated into the sterile basal salt-medium (20 ml, pH 7.5). Culture was agitated at 30°C and 120 rpm for two weeks, after which the suspension was left to stand for 10 min. The supernatant of the culture (500 μl) was transferred to the isolation medium (20 ml) and incubated for 7 days. After several transfers to the isolation medium, the culture solution was spread on an isolation agar medium. The colonies that appeared were distinguished by their color, form and transparency, and all the colonies which seemed to be different were collected and preserved on the slant medium. The OPPEO decomposition activity was examined for each colony and the axenic strains were maintained on an agar slant of the same medium.

Growth test and analysis of biodegradation by the isolated bacteria with OPPEO as the sole carbon and energy source. The isolated bacteria were preliminarily cultured on an agar slant isolation medium. The bacteria were suspended in the isolation medium to make up a bacterial suspension (OD = 1.0). This suspension (1000 μl) was inoculated into a fresh 0.2% OPPEO isolation medium (40 ml), and the medium was agitated at 30°C and 120 rpm. Bacterial growth was monitored by the measurement of the optical density of the medium at 660 nm every 3 hr.

An HPLC analysis was conducted as follows, aliquots 100 μl that were withdrawn every 6 hr was passed through a disposable disk filter (0.2 μm), and 5 μl of the aliquots was analyzed by HPLC.

After 10 days of incubation under the same condition, the culture medium (500 μl) was extracted with an equal volume of ethyl acetate, and the ethyl acetate layer (1 μl) was analyzed by GCMS.

Tentative identification of the degradating bacteria. The bacterial isolates were gram negative and tentatively identified by using the Carbon source utilization system (Biolog; 5) and by a 16S rDNA RFLP analysis.[14,15]

a) The Biolog GN microtiter plates (Biolog Hayworth Co., U.S.A) consisted of 95 substrate-containing wells and one control well without the substrate. Cells freshly grown on a BUGM (Biolog universal growth medium) agar plate were suspended in sterile saline (0.85% NaCl). Immediately after suspending, 150 μl of the suspension per well was inoculated into the Biolog GN microtiter plates. The inoculated Biolog plates were incubated at 30°C. After 1 day of incubation, the results were read with a micro plate reader at a wavelength of 590 nm.

b) Genomic DNA of isolated bacteria was extracted as described by Saito and Miura.[14] The purity of the extracted DNA was checked by the 230:260, and 280:260 nm absorbance ratios. PCR amplification was carried out in a 50-μl volume consisting of 36.6 μl of double distilled water, 5 μl of 10 × PCR buffer, 4 μl of each deoxyribonucleotide triphosphate (2.5 mM), 1 μl of each primer (10 pmol), 0.4 μl of Taq DNA polymerase (5 U ml^{-1}; Takara), and 1 μl of template DNA (0.5 μg to 1.0 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 s, primer annealing at 50°C for 60 s, and primer extension at 72°C for 60 s. The primers were designed from the conserved region of the 16S rDNA sequence of *E. coli*:[15] the forward primer was 5′GCTCAGATTGAACGCTGGCG3′ (41f) corresponding to the 22–41 positions, and the reverse primer was 3′GTCGAGCACAACACTTTACA5′ (1066r) corresponding to the 1066–1085 positions. Ten microliter of the amplified 16S rDNA was digested by selected restriction enzymes and then electrophoretically separated in 2% low-melting-point agarose gel in a Tris-Borate buffer. The gel was stained with ethidium bromide, the fragments pattern on the gel being recorded and the size of the fragments being calculated from a densitogram. DNA fragment sizes smaller than 50 bp were neglected. A similarity search was made by using the program of Watanabe and Okuda.[16,17] This program provides 1533 kinds of 16S rDNA RFLP database consisting of 378 kinds of bacterial genus and 1288 bacterial species.

Results and Discussion

Synthesis of the authentic samples

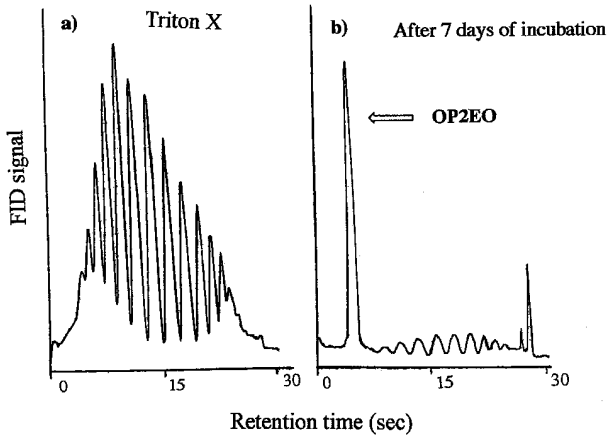

Fig. 2. Iatroscan Chromatogram of OPPEO and Its Biodegradation Products.

Chromatogram a): OPPEO; b): Biodegradation products after 7 days of incubation. Conditions: Stationary phase of Chromarod-S III, mobile phase of ethyl acetate:acetone:H_2O (10:5:1), H_2 gas flow of 160 ml/min, air flow of 2.0 l/min, scanning speed of 30 sec/scan, integrator attenuation of 16. Spotting: a) A 0.2% OPPEO solution was extracted with an equal volume of ethyl acetate. The ethyl acetate layer (3 μl) was spotted on Chromarod-S III. b) The incubation medium (0.5 ml) was extracted with equal volume of ethyl acetate. The ethyl acetate layer (3 μl) was spotted on Chromarod-S III.

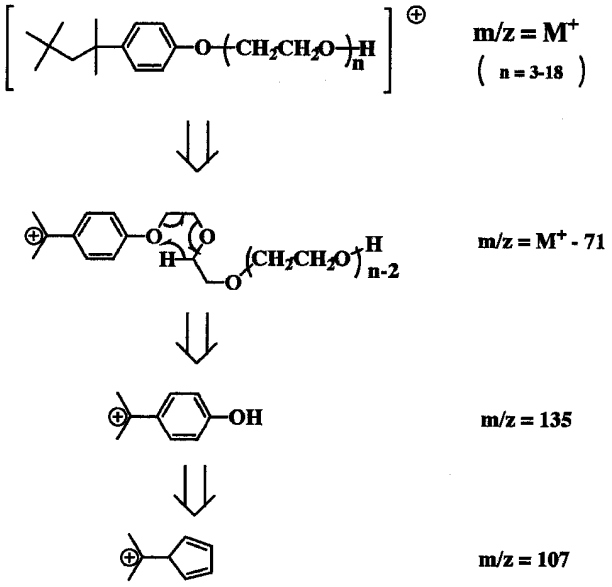

Fig. 3. Typical GCMS Fragmentation Pattern of OPPEO.

Octylphenol was alkylated with ethyl bromoacetate to afford ethyl octylphenoxyacetate in a 78.1% yield, and this phenoxyacetate was reduced with $LiAlH_4$ to give octylphenol monoethoxylate (OP1EO) in an 87.2% yield. The obtained OP1EO was treated with sodium hydride in dry THF, and the resulting alkoxide was alkylate with ethyl bromoacetate to give ethyl 6-[*p*- (1′,1′,3′,3′-tetramethylbutyl)phenyl]-3,6-dioxahexanoate in an 84.4% yield. Reduction of the afforded ester by $LiAlH_4$ gave octylphenol diethoxylate (OP2EO) in a 78.3% yield. Repetition of the same elongation reactions gave OP3EO in a good yield.

Isolation and identification of the OPPEO-degrading bacteria

Each collected soil sample was shaken with the basal salt medium for 7 day and the resulting suspension was extracted with ethyl acetate. The organic layer was spotted on a Chromarod and analyzed by an Iatroscan instrument. Typical chromatograms for OPPEO and the biodegradation products are shown in Fig. 2, the major biodegradation product being identified as OP2EO by comparing with the chromatogram of an authentic sample. Twelve bacterial isolates were obtained by enrichment culture and the subsequent isolation from the soil samples with OPPEO-degrading activity. These isolates were tentatively identified with the Biolog system as *Pseudomonas putida* (10 isolates) and *Burkholderia cepacia* (1 isolate), while the remaining isolate couldn't be identified as shown in Table 1. Most of the 16S rDNA of the 10 strains which had been assigned as *P. putida* by the Biolog system had almost the same RFLP digested by *Hae*III, *Hha* I and *Alu*I (Table 1), and were assigned as *P. putida, P. fulva, P. staminea*, or *P. stuzeri* by the program of Watanabe and Okuda. Although this RFLP analysis was rapid, it proved impossible to distinguish among species with a genetically close relationship. In this case, *P. putida, P. fulva, P. staminea*, and *P. stuzeri* had the same RFLP digested by *Hae*III, *Hha*I and *Alu*I. The strain S1, which was assigned as *B. cepacia*, and strain S11, which was not identified by Biolog, had the same RFLP patterns as those of the *Pseudomonas* group.

GCMS and HPLC analyses of the biodegradation products

The mass spectra of the authentic samples exhibited the typical fragmentation patterns shown in Fig. 3. They afforded small but clear molecular ions, typical M-71 ions resulting from loss of the 2,2-dimethylpropyl radical (usually the base peak) and common m/z 135 ions from loss of the polyoxyethylene side chain by McLafferty rearrangement.

Growth tests were performed on isolated bacteria S-5, S-10 and S-11 with OPPEO as the sole carbon source as shown in Fig. 4. All isolates fed with OPPEO produced an insoluble white precipitate in the culture medium after about 6 hr. With increasing incubation time, the culture grew almost linearly until 21 hr, and then reached stationary phase.

The results of a time-course HPLC analysis on the degradation process of *Pseudomanas putida* (S-10) are shown in Fig. 5. The OP2EO and OP3EO peaks were identified by comparing with those of authentic samples. The bacteria began to degrade OPPEO be-

Table 1. Identification of Isolated Bacteria by Biolog and 16S rDNA RFLP

No.	Soil source	Biolog (*)	Base pair numbers of the restriction enzyme decomposition fragments											Classification by RFLP
			Alu				Hae III			Hha I				
S-1	Yamaguchi 1	*Burkholderia cepacia* (0.901)	387	212	184	160	687	160	100	380	300	242	208	*Pseudomonas sp.*
S-2	Yamaguchi 2	*Pseudomonas putida* (0.988)	406	212	(−)	165	687	160	(−)	381	304	248	211	*Pseudomonas sp.*
S-3	Yamaguchi 3	*Pseudomonas putida* (0.967)	407	213	(−)	165	674	156	(−)	377	304	248	214	*Pseudomonas sp.*
S-4	Fukuoka 1	*Pseudomonas putida* (0.868)	408	220	200	169	700	167	100	351	273	226	192	*Pseudomonas sp.*
S-5	Fukuoka 2	*Pseudomonas putida* (0962)	406	212	(−)	165	688	160	100	378	302	248	211	*Pseudomonas sp.*
S-6	Osaka 1	*Pseudomonas putida* (0.900)	393	208	184	152	700	165	106	376	296	242	205	*Pseudomonas sp.*
S-7	Osaka 2	*Pseudomonas putida* (0.949)	400	208	184	152	700	165	106	371	292	238	205	*Pseudomonas sp.*
S-8	Osaka 3	*Pseudomonas putida* (0.900)	387	212	184	152	688	165	100	372	296	242	208	*Pseudomonas sp.*
S-9	Osaka 4	*Pseudomonas putida* (0.923)	407	220	195	160	685	159	(−)	351	288	245	211	*Pseudomonas sp.*
S-10	Osaka 5	*Pseudomonas putida* (0.951)	407	217	200	165	692	161	(−)	346	277	229	200	*Pseudomonas sp.*
S-11	Osaka 6	N.I.	407	217	195	169	693	164	(−)	346	273	223	192	*Pseudomonas sp.*

* Degree of similarity N.I.: not identified (−): not observed

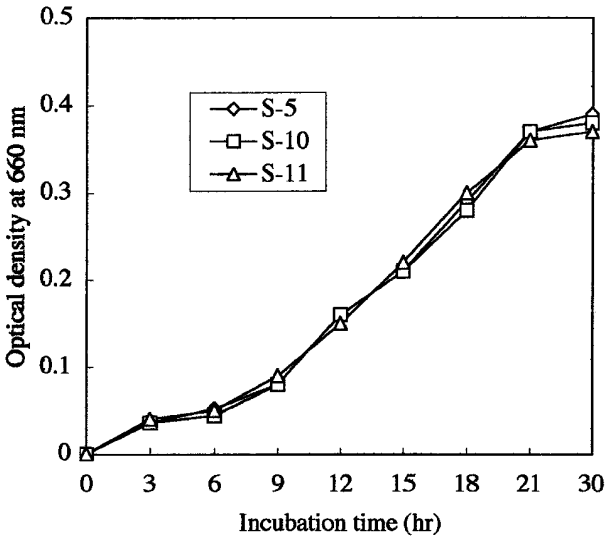

Fig. 4. Growth Curves of the Isolated Bacteria (S-5, S-10 and S-11) in the Presence of OPPEO as the Sole Carbon Source.

Growth was assessed by optical density measurement of the cultures every 3 hr. The culture medium suspension (2 ml) was transferred to an Erlenmeyer flask, and an equal volume of methanol was added to the medium to dissolve the insoluble metabolites. The optical density of the medium was measured at 660 nm.[9]

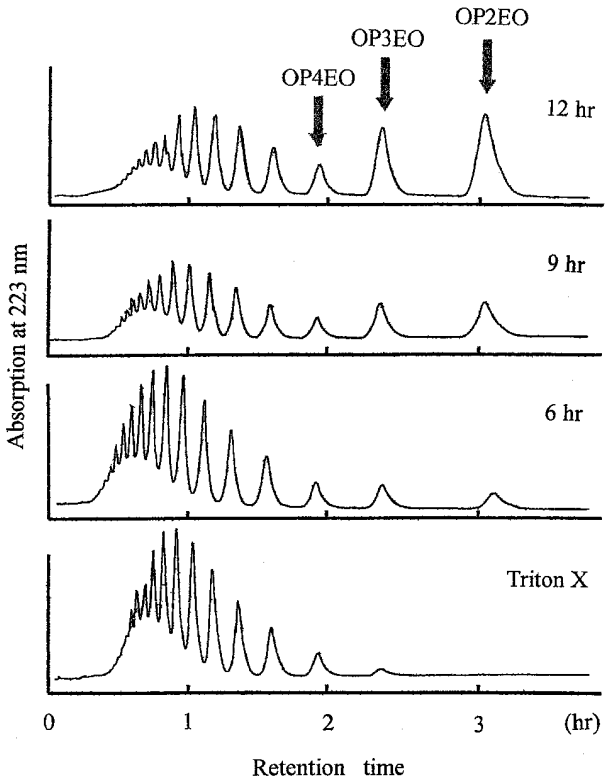

Fig. 5. HPLC Chromatograms of the Time-Course Characteristics for the Degradation Process of OPPEO by *Pseudomonas putida*.

Column, Shodex Mspak GF-310 4E (4.6×250 mm); column temperature, 40°C; solvent, 30% acetonitrile; flow rate, 0.3 ml/min; detection wavelength, 223 nm.

fore 6 hr, whereas the peaks of OP1EO and OP were not apparent even after 12 hr. As the content of octylphenol oligoethoxylate increased with the time, it is obvious that the high-molecular-weight component was degraded to give the low-molecular-weight component and that degradation stopped at OP2EO.

To determine the end product of OPPEO biodegradation, this surfactant was degraded by using each bacterial isolate, and the end products were analyzed by GCMS after 10 days of incubation. All isolates including *Burkholderia cepacia*, gave almost the same GCMS data. The typical total ion chromatogram for S-5 (*Pseudomanas putida*) showed three peaks (a-c) with retention times of 10.57 min (a) and

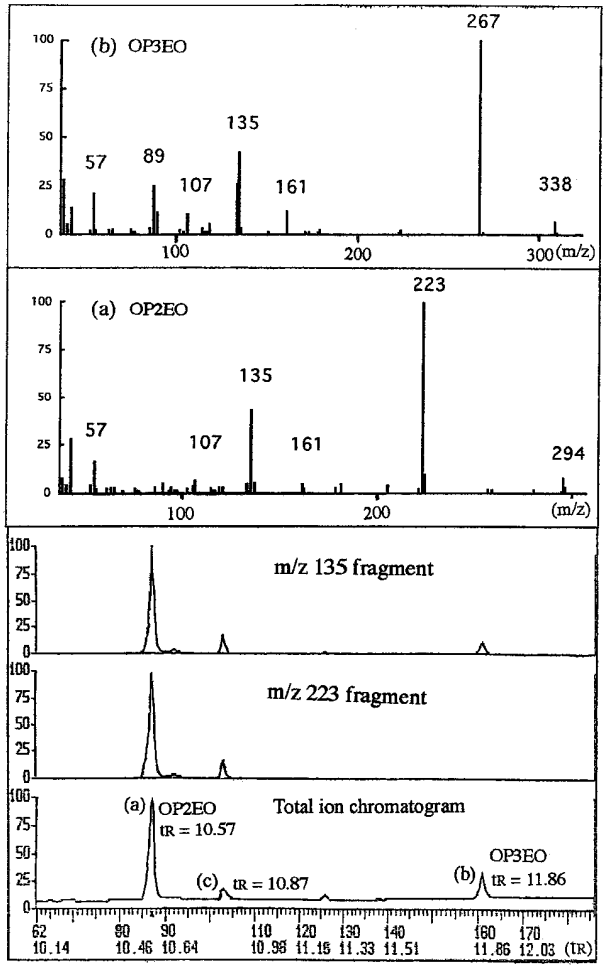

Fig. 6. Total Ion Chromatogram and the Mass Spectra of the OPPEO Biodegradation Products by *Pseudomonas putida*.

The culture medium (1 ml) was transferred to an Eppendorf tube and extracted with HPLC-grade ethyl acetate (1 ml) by vortex mixing for 5 min. The ethyl acetate layer (500 μl) was transferred to a new tube and centrifuged at 8000 rpm for 5 min. The supernatant (400 μl) was diluted with 1600 μl of ethyl acetate, and the resulting solution was analyzed by a Perkin Elmer Q-910 GCMS instrument fitted with a capillary column (SPD^{TM}1, 30 m by 0.32 mm) and an automated injector. The GC conditions were as follows: injection volume, 3 μl; carrier gas, helium at 8.0 *psi*; temperature gradient, 100°C isothermal for 3 min, 100–300°C gradient at 20°C/min, 300°C isothermal for 10 min; total running time, 25 min. The voltage of the electron multiplier and the ionizer temperature were 1100 V and 300°C, respectively.

11.86 min (b). The spectra for (a) and (b) gave the M^+ ions at 294 and 338, corresponding to OP2EO and OP3EO, respectively (Fig. 6). Typical peaks (M-71) resulting from the loss of the 2,2-dimethylpropyl group were the base peaks of OP2EO and OP3EO, respectively. Confirmation of their identity was provided by comparing the retention times and fragmentation patterns of OP2EO and OP3EO with those of authentic samples. The total ion chromatogram showed another peak (c) whose retention time was 10.87 min. In the spectrum for (c), the M^+ ion is at 294, and its fragmentation pattern is similar to that of OP2EO. We have assumed that metabolite c must have been a positional isomer of OP2EO, *i.e.* the *ortho* isomer. This result indicates that the extract did not contain any OP1EO and OP since there existed no peak with a shorter retention time than that of OP2EO. It was revealed that all the isolated bacteria degraded OPPEO from the end of the polyethoxy chain and left mainly OP2EO in the culture medium under aerobic conditions. Since OPPEO itself has low toxicity and is not estrogenic,[18] it has been widely used as a wetting and dispersing additive in pesticide formulations. It is, however, difficult to know the actual conditions of use of OPPEO, since there is no obligation to indicate OPPEO use. The results of this present work indicate that the aerobic microorganisms in paddy fields could degrade OPPEO to endocrine-disrupting OP2EO and OP3EO. It therefore seems necessary to investigate the formation of OP2EO and OP3EO.

Acknowledgments

This work was supported in part by a Grant-in-aid (Endocrine Disrupters) from the Ministry of Agriculture, Forestry, and Fisheries of Japan (ED-02-V-1-1).

References

1) Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N., and Serrano, F. O., The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ. Health Perspect.*, **103** (Suppl 7), 113–122 (1995).
2) Klotz, D. M., Beckman, B. S., Hill, S. M., McLachlan, J. A., Walters, M. R., and Arnold, S. F., Identification of environmental chemicals with estrogenic activity using a combination of *in vitro* assays. *Environ. Health Perspect.*, **104**, 1084–1089 (1996).
3) Korner, W., Hanf, V., Schuller, W., Bartsch, H., Zwirner, M., and Hagenmaier, H., Validation and application of a rapid *in vitro* assay for assessing the estrogenic potency of halogenated phenolic chemicals. *Chemosphere*, **37**, 2395–2407 (1998).
4) Jobling, S., Reynolds, T., White, R., Parker, M. G., and Sumpter, J. P., A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environ. Health Perspect.*, **103**, 582–587 (1995).
5) Tabira, Y., Nakai, M., Asai, D., Yakabe, Y., Tahara, Y., Shinmyozu, T., Noguchi, M., Takatsuki, M., and Shimohigashi, Y., Structural requirements of *para*-alkylphenols to bind to estrogen receptor. *Eur. J. Biochem.*, **262**, 240–245 (1999).
6) Nagel, S. C., von Saal, F. S., Thayer, K. A., Dhar, M. G., Boechler, M., and Welshons, W. V., Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative *in vivo* bioactivity of the xenoestrogens bisphenol A and octylphenol. *Environ. Health Perspect.*, **105**, 70–76 (1997).

7) Laws, S. C., Carey, S. A., Ferrell, J. M., Bodman, G. J., and Cooper, R. L., Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. *Toxicol. Sci.*, **54**, 154–167 (2000).
8) Dominic, M. J., and White, G. F., Mechanism for biotransformation of nonylphenol polyethoxylates to xenoestrogens in *Pseudomonas putida*. *J. Bacteriology*, **1998**, 4332–4338 (1998).
9) Scott, M. J., and Jones, M. N., The biodegradation of surfactants in the environment. *Biochim. Biophys. Acta*, **1508**, 235–251 (2000).
10) Ferguson, P. L., Iden, C. R., and Brownawell, B. J., Analysis of alkylphenol ethoxylate metabolites in the aquatic environment using liquid chromatography-electrospray mass spectrometry. *Anal. Chem.*, **72**, 4322–4330 (2000).
11) Petrovic, M., and Barcelo, D., Determination of anionic and nonionic surfactants, their degradation products, and endocrine-disrupting compounds in sewage sludge by liquid chromatography/mass spectrometry. *Anal. Chem.*, **72**, 4560-4567 (2000).
12) Tsuda, T., Takino, A., Kojima, M., Harada, H., Muraki, K., and Tsuji, M., 4-Nonylphenols and 4-*tert*-octylphenol in water and fish from rivers flowing into Lake Biwa. *Chemosphere*, **41**, 757–762 (2000).
13) Bolz, U., Korner, W., and Hagenmaier, H., Development and validation of a GC/MS method for determination of phenolic xenoestrogens in aquatic samples. *Chemosphere*, **40**, 929–935 (2000).
14) Saito, H., and Miura, T., Preparation of transforming DNA by phenol treatment. *Biochim. Biophys. Acta*, **72**, 619–629 (1963).
15) Brosius, J., Palmer, M. L., Kennedy, P. J., and Noller, H. F., Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*, **75**, 4801–4805 (1978).
16) Watanabe, K., and Okuda, M., Japan Kokai Tokkyo Koho, Registration No. 2000–215134 (July 14, 2000).
17) Watanabe, K., and Okuda, M., U.S. Patent, Method and System for Searching for Relationships between Base Sequences in Genes. Registration No. 24618 (July 10, 2001).
18) White, R., Jobling, S., Hoare, S. A., Sumpter, J. P., and Parker, M. G., Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology*, **135**, 175–182 (1994).