Short Communication

Isolation of Octylphenol Polyethoxylate-Degrading Soil Bacteria: a Long-Term Soil Column Study

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A soil column was percolated with Triton X-100 (octylphenol polyethoxylates; OPEOₙ) under aerobic conditions for 300 days and OPEOₙ-degrading bacteria were isolated and characterized phylogenetically. Populations of total culturable bacteria and OPEOₙ-resistant bacteria increased 10 times and 46 times, respectively, during the percolation. 16S rRNA gene sequencing of 128 OPEOₙ-resistant isolates revealed an increase in the population level of Alphaproteobacteria and decreases in levels of Betaproteobacteria and Actinobacteria with the exposure to the compound. Four OPEOₙ-degrading strains were isolated and characterized as being related to Bradyrhizobium liaoningense (3 strains) and Afipia sp. (1 strain).

Key words: octylphenol polyethoxylates, soil percolation column, Bradyrhizobium liaoningense, Afipia sp.

Alkylphenol polyethoxylates (APEOₙ; n, number of ethoxy units) such as octylphenol polyethoxylates (OPEOₙ) and nonylphenol polyethoxylates (NPEOₙ) are widely used as non-ionic surfactants in cleaning products and as industrial aids: the spectrum of applications ranges from dispersing agents in paper and pulp production to emulsifying agents in latex paints and pesticide formulations, flotation agents, industrial cleaner, and household cleaners. The majority of APEOₙ are used in aqueous solutions; therefore they are discharged into municipal and industrial waste waters. Elucidation of the primary mechanisms of biodegradation of APEOₙ is important to understand the fate of APEOₙ in the natural environment. The three most common groups of APEOₙ degradation intermediates reported are as follows: (a) alkylphenols (e.g., nonylphenol and octylphenol); (b) short-chain APEOₙ having one to four ethoxylate units with alkylphenol diethoxylates as the predominant intermediate; and (c) a series of ether carboxylates including alkylphenoxy acetic acid and alkylphenoxyethoxyacetic acid. Fewer studies have focused on OPEOₙ than on NPEOₙ. Two studies showed that OPEOₙ underwent degradation similar to NPEOₙ under aerobic and anaerobic conditions of wastewater treatment and in an upflow anoxic sludge blanket reactor. Some pure culture studies have been also conducted on the degradation of OPEOₙ by specific bacteria, and several conventional batch enrichments were performed with samples from a surfactant-polluted site of a pesticide factory, paddy field soils, and golf course soils to isolate bacteria capable of degrading OPEOₙ. The strains from the three studies were identified as Pseudomonas in the Gammaproteobacteria and one strain of the other study as Sphingomonas in the Alphaproteobacteria. However, little information is available on the degradation of OPEOₙ in the soil environment and in situ changes in the soil microbial community with long exposure to the chemicals. The
aim of the present study was to investigate soil bacterial populations in response to long-term exposure to \( \text{OPEO}_n \).

To this end, a soil column system was percolated continuously with \( \text{OPEO}_n \) for 300 days. Thereafter, bacteria able to degrade \( \text{OPEO}_n \) were isolated and characterized phylogenetically. The initial loading concentration of \( \text{OPEO}_n \) was set at 800 mg L\(^{-1}\), comparable to levels (1,000–2,000 mg L\(^{-1}\)) in the above batch enrichment experiments.

The soil used in this study was sampled from arable land at the Agricultural Experimental Station, Ibaraki University College of Agriculture at a depth of 0 to 20 cm, sieved (<2 mm), and kept at 4°C before the soil percolation study. In the arable field, yacon plants (\( \text{Polymnia sonchifolia} \)) had been cultivated before soil sampling. The soil column consisted of a glass cylinder (inner diameter, 30 mm; length, 250 mm) and was prepared by introducing 25 g of soil on a glass wool layer at the bottom of the column. A glass wool layer was also placed on the surface of the soil column to allow even spreading of the input solution through the column. The circulation of percolating solution was carried out by a peristaltic pump (PERISTA\(^{\circledR}\) Bio-minipump, EYELA, Tokyo, Japan) and the input solution was applied to the top of the column at a flow rate of 0.16 ml min\(^{-1}\). Air was supplied to the columns by an aeration pump (Aeration MAU-1, EYELA) at a flow rate of 10 ml min\(^{-1}\). Five hundred milliliters of percolating solution (containing 800 mg L\(^{-1}\) of \( \text{OPEO}_n \)) was added to a 1000-ml Erlenmeyer flask and used as a reservoir. Glass tubing was fixed to the bottom of the glass column, which reached into the percolating fluid reservoir. During percolation, the soil column was kept at 30°C. \( \text{OPEO}_n \) (Triton X-100) with 98% purity and 4-\( \text{t}-\)octylphenol (OP) were purchased from Sigma Aldrich (Milwaukee, USA). The \( \text{OPEO}_n \) contain a branched chain octylphenol attached to a polyethoxylate chain with an average of around 9.5 ethoxylate units. All other regents of chemical analysis were purchased from Wako Pure Chemical Industries (Osaka, Japan).

The dilution-plating method was used to enumerate culturable bacteria using 100-fold diluted nutrient broth (NB/100) as the culture medium\(^{4-24}\). Soil samples (5 g) were taken from the column before percolation (0 day) and at 129 and 300 days after exposure to \( \text{OPEO}_n \), suspended in 45 ml of sterile water, dispersed on an EYELA ultrasonic cleaner for 5 min, then diluted in a 10-fold stepwise manner with sterile water. The dilutions were inoculated on NB/100 agar plates, NB/100 agar supplemented with 1000 mg L\(^{-1}\) \( \text{OPEO}_n \), and 10-fold diluted NB (NB/10)\(^9\) agar supplemented with 200 mg L\(^{-1}\) OP. Cultivable bacteria were enumerated after incubation at 30°C for 4 weeks then isolated from the plates. Bacteria grown on the \( \text{OPEO}_n \)- and OP-supplemented media were regarded as being \( \text{OPEO}_n \)-resistant and OP-resistant, respectively. The isolation procedure was essentially the same as previously described\(^{23,24}\) and bacteria were maintained at room temperature in semisolid DNB stab cultures. Pure cultures of isolates were examined with regard to cell morphology and Gram staining as described by Ohta and Hattori\(^{23}\). For further phenotypical characterization, the API 20 NE kit system (bioMérieux, Marcy l’Etoile, France) was used according to the recommendations of the manufacturer with \( \text{Bradyrhizobium liaonensis} \) NBRC 100396 as a reference strain.

For the phylogenetic analysis of bacterial strains, genomic DNA was extracted from bacterial cells grown on DNB agar plates by the method of Wang and Wang\(^{31}\) and used as the template for amplification of the 16S rDNA gene (16S rDNA) via PCR with the primers 10F (5’-\text{AGTTTGATCCTGGCTCAG-3’}, corresponding to positions 10–27 of the \text{Escherichia coli} 16S rDNA) and 1541R (5’-\text{AAGGAGGTGATCCAGCCG-3’}, positions 1524–1541). The PCR condition, the electrophoresis of amplified DNA, and the purification of PCR products were essentially the same as described previously\(^{4-29}\).

Nucleotide sequences were determined with a Taq Dye Deoxy Terminator Cycle Sequencing kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) and read on an Applied Biosystems 3100 DNA sequencer. The primers used for the sequencing of \( \text{OPEO}_n \)-resistant isolates were 10F and 1541R, and those for \( \text{OPEO}_n \)-degrading strains were as follows: 10F, 800F (5’-\text{ATTTGTATCTGGCTCAG-3’}, positions 800–816), 786R (5’-\text{GACTACCAGGGTATCTAATC-3’}, positions 767–800), and 1541R. All sequences determined in this way were compared with similar DNA sequences retrieved from the DDBJ/EMBL/GenBank databases using the BLAST program\(^{25}\). For the phylogenetic analysis of the sequence datasets, the CLUSTAL W program\(^{26}\) was utilized and a phylogenetic tree was constructed by the neighbor-joining method\(^{26}\). The 16S rDNA sequence data for isolates in this study have been deposited under DDBJ accession numbers AB269542 to AB269669.

The modified protocol established by John and White\(^{17}\) for the cobalt thiocyanate active substance (CTAS) assay\(^{30}\) was used to determine the amount of \( \text{OPEO}_n \) in the percolating solution. In brief, aliquots (1 ml) of percolation fluids taken from the percolation reservoir were centrifuged and mixed with 1.25 ml of ammonium cobalt thiocyanate solution, 1.5 ml of chloroform, and 0.8 g of NaCl. The mixtures were vortex-mixed for 5 min and the absorbance of the chloroform layer was measured at 320 nm on a Shimadzu
(Kyoto, Japan) UV-1000 spectrophotometer. To determine several OPEO homologues, aliquots of percolation fluids were also analyzed by atmospheric pressure chemical ionization (APCI)-liquid chromatography-tandem mass spectrometry (LC/MS/MS). The APCI-LC/MS/MS system was composed of an Agilent Technologies (Palo Alto, CA, USA) 1100 HPLC instrument and an Applied Biosystems PE Sciex API 300 MS. The samples were separated on an Inertsil ODS-2 Column (150 mm × 4.6 mm, i.d., 5 µm; GL Science, Tokyo, Japan). A binary mobile phase with 0.1% formic acid in distilled water (20%) and 0.1% formic acid in acetonitrile (80%) was used at a flow rate of 1.0 ml min⁻¹.

OPEOs were analyzed in the positive ion mode with nitrogen being used as the collision gas. The following transitions from precursor to product ions were monitored in the multiple reaction monitoring (MRM) mode: OPEO₇⁻→133.0; OPEO₈, 559.4→133.0; OPEO₉, 603.4→133.0; OPEO₁₀, 647.4→133.0; OPEO₁₁, 691.5→133.0; OPEO₁₂, 735.5→133.0.

OPEO₇-resistant isolates were tested for their ability to degrade OPEO₇ using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) as the coupling agent with the degradation of OPEO₇ as described by Hatzinger et al.¹¹ and Hongwei et al.¹² The growth medium (NB/100) was supplemented with 200 µM INT and bacteria were grown for 2 weeks in microtiter wells containing 200 µl of the medium and 100 mg L⁻¹ OPEO₇, and wells containing 200 µl of the medium only. The reduction of INT was assayed by measuring the absorbance at 490 nm on a Wallac Arvo SX 1420 multilabel counter (Perkin Elmer Applied Biosystems). Cultures exhibiting significant increases in the reduction with the addition of OPEO₇ were assumed to be positive for the degradation of OPEO₇. Subsequently, the INT assay-positive strains were grown in flasks containing NB/100 (50 ml) supplemented with 100 mg L⁻¹ of OPEO₇, with shaking (300 rpm) at 30°C for 4 weeks: OPEO₇ was added at 500 and 1000 mg L⁻¹ in some growth experiments and growth was followed by measuring the turbidity at 660 nm (OD₆₆₀) on the Shimadzu UV-1000 spectrophotometer. Aliquots of cultures were centrifuged, diluted with methanol, and analyzed with the APCI-LC/MS/MS system.

OP-resistant isolates were grown on 5-fold diluted NB (NB/5) agar medium supplemented with 100 mg L⁻¹ OP at 30°C for 2 weeks. The ability to degrade OP was judged from changes in the color of the medium from being turbid to yellowish transparent as reported previously (Ohtsuka, M., Y. Iknaga, M. Hasegawa, Y. Kurusu, and H. Ohta, “Degradation of octylphenol by Sphingobium amiense YT: effects of cyclodextrin on the degradation”, in the 21st Annual Meeting of the Japanese Society of Microbial Ecology, abstract No. PE-65, 2005). OP-resistant strains were further tested by growing in NB/5 liquid medium (supplemented with 100 µg ml⁻¹ OP and 50 µl L⁻¹ of 10% cyclodextrin as the agent masking the strong lipophilicity of OP. Cultures were incubated with shaking (300 rpm) for 10 days at 30°C and intermediates of OP degradation were analyzed by gas chromatography-mass spectrometry (GC-MS). For the GC-MS sample preparation, aliquots (500 µl) of cultures were mixed with an equal volume of ethyl acetate (10 mg L⁻¹ butyl phenol in ethyl acetate) and 50 µl of 1 M HCl. The ethyl acetate layer sampled was dried with N₂ gas. Dried samples were redisolved in BSTFA+TMCS (99:1) (Supelco, Bellefonte, PA, USA) prior to GC-MS analysis.

The GC/MS conditions were as follows: GC, 6980N Network GC system (Agilent Technologies); MS, JMS-BU25 GC Mate II (JEOL Ltd., Tokyo, Japan); column, HP-5, 30 m×0.25 mm, 0.25 µm-film (J & W Scientific, Inc., Folsom, CA, USA); carrier gas, helium at a flow rate of 1 ml min⁻¹; GC temperature program, 55°C for 3 min, 6°C min⁻¹ to 200°C, 15°C min⁻¹ to 295°C, and 295°C for 3 min; injection volume, 1 µl; ionization mode, electron ionization (70 eV); scan range m/z, 40–600.

The percolation liquid was sampled periodically from the reservoir and the amount of OPEO₇ in the solution was determined by CTAS assay (Fig. 1). The concentration of OPEO₇ decreased exponentially and the profile of this decline was divided into two phases. The first phase was divided into two phases: the first phase (0–120 days), c=820·e⁻⁰·⁰¹⁹⁶ (r=0.988); the second phase (120–300 days), c=280·e⁻⁰·⁰⁰⁰₈ (r=0.681), where c and r are the concentration of octylphenol polyethoxylates (mg L⁻¹) and time (days), respectively.
between days 0 and 120 and the rate of decrease approximated a first-order reaction at \(-0.011\) day\(^{-1}\) \((r=0.988)\). The second phase occurred between days 120 and 300 with a rate constant of \(-0.0013\) day\(^{-1}\) \((r=0.681)\). The APCI-LC/MS/MS analysis revealed that the degradation of OPEO\(_{11,12}\) also occurred in a two-phase manner and their degradation time courses were classified into 3 profiles. OPEO\(_{12}\) underwent degradation at higher rates in the first phase (average rate constant, \(-0.017\) day\(^{-1}\)) than in the second phase (\(-0.008\) day\(^{-1}\)). In the case of OPEO\(_{11,12}\), the degradation rates were higher in the second phase (average rate constant, \(-0.018\) day\(^{-1}\)) than in the first phase (\(-0.010\) day\(^{-1}\)). The degradation rate of OPEO\(_{10}\) in the first phase (rate constant, \(-0.014\) day\(^{-1}\)) was similar to that in the second phase (\(-0.013\) day\(^{-1}\)). In parallel with the decrease of OPEO\(_{n}\), the populations of total culturable bacteria and OPEO\(_{n}\)-resistant bacteria increased 10 times and 46 times, respectively, during the soil percolation performance for 300 days (Table 1). The specific increase of OPEO\(_{n}\)-resistant bacteria in the total bacterial population was also indicated by calculating the percentage: 6.9% before percolation with OPEO\(_{n}\) versus 34–35% after the percolation. The degradation of OPEO\(_{n}\) was expected to result in the formation of OP, which might lead to an increase in the population of OP-utilizing bacteria. Therefore assuming that bacterial utilization of OP involves resistance to OP, bacteria resistant to 200 mg L\(^{-1}\) OP were enumerated. As shown in Table 1, the population level was not dependent on the percolation with OPEO\(_n\), suggesting no significant increase in OP-degrading populations.

A total of 128 strains of OPEO\(_{n}\)-resistant bacteria were isolated from the soil in the column before percolation with OPEO\(_{n}\) (20 isolates) and at 129 days (23 isolates) and 300 days (85 isolates). From the 16S ribosomal RNA gene sequence analysis, 60% and 83–84% of strains isolated before and after the percolation, respectively, were affiliated with Alphaproteobacteria. Another group enhanced by OPEO\(_n\) loading was Gammaproteobacteria, which increased from 5% of isolates before percolation to 9% and 13% at 129 and 300 days, respectively. In contrast, the population of Betaproteobacteria decreased from 25% to 4% and that of Actinobacteria decreased from 10% to <1.2% with OPEO\(_n\) loading. A phylogenetic tree including these OPEO\(_{n}\)-resistant bacteria and related strains is shown in Fig. 2. Among the strains isolated from the soil after 300 days of percolation, 20 had almost identical 16S rRNA gene sequences (about 650 nucleotides; representative strain, ARP-G8). The almost complete 16S rRNA gene sequence (1479 nucleotides) of strain ARP-G8 was determined and its closest relatives were the sequences of Afipia sp. strain SP17 with an identity value of 98.5%, Oligotropharpha carboxidovorans strain S23 (98.3%), and Afipia broomeae strain C-12 (98.2%). The second group (15 strains; representative strain, ARP-C1) was more closely related to O. carboxidovorans strain S23 with a sequence identity value of 99.1%. The third group (11 strains; representative strain, ARP-A3) showed sequence identity values of 99.7% with the sequence of Afipia broomeae strain G7472.

Representative OPEO\(_{n}\)-resistant strains listed in Fig. 2 were examined for the degradation of OPEO\(_{n}\) and finally four strains (ARP-2-16, ARP-A1, ARP-D6, and ARP-G8) were found to be clearly positive for the degradation. In the degradation assay, changes in relative amounts of OPEO\(_{11,12}\) in the 100 mg L\(^{-1}\) Triton X-100-containing cultures were followed during 28 days’ incubation. As shown in Fig. 3, the rate of decrease was higher for the shorter-chain homologues (OPEO\(_{7,8}\)) than longer-chain homologues (OPEO\(_{11,12}\)) in all cultures. Strains ARP-D6 and ARP-G8 were further examined for their utilization of OPEO\(_n\) by measuring growth yields of cultures in the presence of different amounts of OPEO\(_n\). NB/100 was used as the basal growth medium and OPEO\(_n\) was added at final concentrations of 100, 500, and 1000 mg L\(^{-1}\). The growth rate was increased from 0.017 h\(^{-1}\) to 0.040 h\(^{-1}\) (strain ARP-D6) and from 0.010 h\(^{-1}\) to 0.043 h\(^{-1}\) (strain ARP-G8) by addition of 100 mg L\(^{-1}\) Triton X-100. However, further increases of the OPEO\(_n\) concentration to 500 and to 1000 mg L\(^{-1}\) did not influence the growth rate: 0.018 h\(^{-1}\) at 500 mg L\(^{-1}\) and 0.019 h\(^{-1}\) at 1000 mg L\(^{-1}\) for strain ARP-D6 and 0.018 h\(^{-1}\) at 500 mg L\(^{-1}\) and 0.026 h\(^{-1}\) at 1000 mg L\(^{-1}\) for strain ARP-G8. Growth yield was estimated from total growth, i.e., the difference between the OD\(_{660}\) of the culture at the stationary phase and the OD\(_{660}\) just after inoculation. Total growth was slightly suppressed by the addition of OPEO\(_n\). 0.110 at 0 mg L\(^{-1}\) versus 0.082, 0.091, and 0.088 at 100, 500, and 1000 mg L\(^{-1}\), respectively, for strain ARP-D6 and 0.125 at 0 mg L\(^{-1}\).

**Table 1.** Populations of culturable bacteria in the OPEO\(_{n}\)-loaded soil column

<table>
<thead>
<tr>
<th>Medium</th>
<th>Log CFU/g dry soil (percentage of CFU)</th>
<th>Incubation period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>NB/100</td>
<td>9.1 (100)</td>
<td>10.2 (100)</td>
</tr>
<tr>
<td>NB/100 plus 1000 mg L(^{-1}) OPEO(_{10})</td>
<td>7.9 (6.9)</td>
<td>9.7 (34)</td>
</tr>
<tr>
<td>NB/10 plus 200 mg L(^{-1}) OP</td>
<td>7.0 (0.8)</td>
<td>8.0 (0.7)</td>
</tr>
</tbody>
</table>
Fig. 2. Neighbor-joining phylogenetic tree for OPEO₈-resistant isolates and their phylogenetic relatives, based on the nucleotide sequence of the 16S rRNA gene. Bacillus subtilis NCDO 1769³ is used as the outgroup to root the tree. Strains designated as ARP are OPEO₈-resistant isolates and those from the soils with 0, 129, and 300 days' exposure to OPEO are marked with an asterisk, an open square, and a closed square, respectively. The numbers in the parentheses show the numbers of strains possessing complete or almost-complete sequences of representative strains. Nodes indicating >85% and 50–84% bootstrap values (1000 resamplings) are shown by closed and open circles, respectively.
versus 0.110, 0.106, and 0.095 at 100, 500, and 1000 mg L\(^{-1}\), respectively, for strain ARP-G8. These results indicate that these strains were not able to utilize OPEO\(_n\) as a growth substrate. With respect to the stimulative effect of OPEO\(_n\) on the growth rate, Chen et al.\(^7\) reported that five OPEO\(_n\)-degrading *Pseudomonas* sp. strains cultured with different amounts of OPEO\(_n\) as the sole carbon source showed an optimum growth rate at 100–10,000 mg L\(^{-1}\). Although the authors did not provide the growth yield data, the growth enhancement seemed to be linked with the utilization of the compounds. In the case of our strains, no satisfactory explanation is currently available for the increase in the growth rate with 100 mg L\(^{-1}\) OPEO\(_n\).

With respect to the utilization of OP, the above four strains (ARP-2-16, ARP-A1, ARP-D6, and ARP-G8) showed negative reactions for the screening assay measuring changes in the color of the medium from being turbid to yellowish transparent. Among the representative strains listed in Fig. 2, only one strain (ARP-C6) showed a weakly positive reaction in the screening assay. However, the subsequent GC/MS analysis revealed that the amount of OP was not changed and degradation intermediates were not detected in the OP-supplemented culture of the strain, indicating no degradation activity of this strain.

From the 16S rRNA gene sequence analysis (about 1480 nucleotides), the closest relative of our three OPEO\(_n\)-degrading strains (ARP-2-16, ARP-A1, and ARP-D6) is *Bradyrhizobium liaoningense* strain LYG10 with an identity value ranging from 97.8% (ARP-2-16) to 99.3% (ARP-D6). The other strain (ARP-G8) is related to *Afipia* sp.
strain SP17 (98.5%) and O. carboxidovorans strain S23 (98.3%) in the family Bradyrhizobiaceae. Because strains of B. liaoningense utilize a narrow range of organic compounds, our four strains were characterized with the API 20NE kit system. Their phenotypic characteristics were very similar to each other and also to those of B. liaoningense NBRC 100396: the cells were Gram-negative and all were positive for urease and negative for nitrate reduction, indole production, escurin hydrolysis, gelatine hydrolysis, and ß-galactosidase. The strains including B. liaoningense NBRC 100396 did not utilize glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate, and phenyl acetate. Only one difference was found in the production of arginine dihydrolase which was positive in strain ARP-D6 and negative in the other strains. To the best of our knowledge, the activity of OPEO degradation is not described in any studies on B. liaoningense. Further characterization of our B. liaoningense-related strains is now in progress in the laboratory. Strain ARP-G8 was phylogenetically related to O. carboxidovorans strain S23 which had been isolated from sewage-activated sludge (Kawada Sewage Treatment Plant, Utsunomiya, Japan). The presence of surfactants normally occurs in sewage treatment plants and this may explain the enrichment of the O. carboxidovorans strain S23-related strain by the long-term exposure to OPEO in the soil column.

A number of bacterial strains able to degrade OPEO or NPEO have been isolated from soil and aquatic environments. They were identified as mainly Gammaproteobacteria and one of the strains described by Nishio et al. was identified as Sphingomonas macrogoltabidus in the Alphaproteobacteria. In contrast to those previous reports, our long-term soil percolation experiment yielded the isolation of bacterial strains belonging to the family Bradyrhizobiaceae in the Alphaproteobacteria. An explanation for the isolation of different bacterial taxa may be the OPEO concentration used in our soil percolation experiment [0.08% (w/v)], which was slightly lower than in the case (0.2%) of the conventional enrichment of Pseudomonas putida in paddy field soils. However, it is more likely that the predominant bacterial species in response to long-term exposure to OPEO (10 months) will be different from those isolated by normal short-term enrichments (<1 month). Our results also suggest that soil bradyrhizobia have physiological and biochemical properties resulting in a selective advantage in terms of exposure to surfactants such as OPEO.

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References