Appl Microbiol Biotechnol (2006) 72: 552–559
DOI 10.1007/s00253-005-0288-z

Xin Liu · Akio Tani · Kazuhide Kimbara · Fusako Kawai

Metabolic pathway of xenoestrogenic short ethoxy chain-nonylphenol to nonylphenol by aerobic bacteria, *Ensifer* sp. strain AS08 and *Pseudomonas* sp. strain AS90

Received: 2 November 2005 / Revised: 28 November 2005 / Accepted: 1 December 2005 / Published online: 10 March 2006

Abstract *Ensifer* sp. strain AS08 and *Pseudomonas* sp. strain AS90 degrading short ethoxy (EO) chain-nonylphenol (NP) [$NPEO_{av2.0}$ containing NP mono- ~ tetraethoxylates (NP1EO ~ NP4EO); average 2.0 EO units] were isolated by enrichment cultures. Both strains grew on NP but not on octyl- and nonylphenol polyethoxylates (NPEOs) (average 10 EO units). Growth and degradation of $NPEO_{av2.0}$ was increased with increased concentrations of yeast extract (0.02–0.5%) in a culture medium. Culture supernatants of both strains grown on $NPEO_{av2.0}$ were analyzed by high-performance liquid chromatography, showing degradation of NP4EO–NP1EO. The metabolites from nonylphenol diethoxylate (NP2EO) by resting cells of both strains were identified by gas chromatography–mass spectrometry as nonylphenoxyethoxyacetic acid, NP1EO, nonylphenoxy-acetic acid (NP1EC), and NP, while those from NP1EO were identified as NP1EC and NP. Cell-free extracts from strain AS08 grown on $NPEO_{av2.0}$ dehydrogenated NPEOs, $NPEO_{av2.0}$, NP2EO, NP1EO, and PEG 400, but the extracts were inactive toward di- ~ tetraethylene glycol. Aldehydes were formed in the reaction mixture of each substrate with cell-free extracts. From these results, the aerobic metabolic pathway for short EO chain-NP is proposed: A terminal alcohol group of the EO chain is oxidized to a carboxylic acid via an aldehyde, and then one EO unit is removed. This process is repeated until NP is produced.

English edition: The paper was edited by a native speaker through KN international (http://www.kninter.com).

X. Liu · A. Tani · K. Kimbara · F. Kawai (✉)
Laboratory of Applied Microbiology,
Research Institute for Bioresources,
Okayama University, Kurashiki, 2-20-1 Chuo,
Kurashiki 710-0046, Japan
e-mail: fkawai@rib.okayama-u.ac.jp
Tel.: +81-86-4341225
Fax: +81-86-4341225
e-mail: dns14517@cc.okayama-u.ac.jp
e-mail: atani@rib.okayama-u.ac.jp
e-mail: kimbara@rib.okayama-u.ac.jp

Introduction

Nonylphenol polyethoxylates (NPEOs) belong to a group of nonionic surfactants that are used on a large scale worldwide in both industry and the household. They are often found as pollutants in natural aquatic environments and in raw municipal wastewater. The primary aerobic degradation of NPEOs in wastewater treatment plants (WWTPs), in water environments, and by pure cultures (Ahel et al. 1987; Planas et al. 2002; Kvestak and Ahel 1994; Maki et al. 1994; John and White 1998) produced more recalcitrant metabolites, such as nonylphenol diethoxylate (NP2EO) and nonylphenol monoethoxylate (NP1EO), as well as nonylphenoxyethoxyacetic acid (NP2EC) and nonylphenoxyacetic acid (NP1EC). These metabolites were further transformed to nonylphenol (NP) under anaerobic conditions (Giger et al. 1984; Ejlertsson et al. 1999), but there is no report thus far on the transformation to NP under aerobic conditions. These metabolic compounds have recently attracted scientists' concern not only because they possess stronger toxicity than their precursors (Patoczka and Pulliam 1990), but also because evidence is mounting that they are potential endocrine disrupters (Jobling and Sumpter 1993; Soto et al. 1991). As NP, NP1EO, and NP2EO are lipophilic, they tend to accumulate easily in sediments and aquatic organisms. Thus, they are hazardous to human beings through food chains.

Many reports have described methods for eliminating these estrogenic xenobiotics on a large scale, such as in WWTPs (Wintgens et al. 2003) or in fields (Jonkers et al. 2003; Montgomery-Brown et al. 2003), or on a laboratory scale (Jonkers et al. 2001) with sewage sludge, soil consortia, or sediment samples from polluted rivers or lakes. Some microorganisms possess the ability to as-

similate NP as a sole carbon and energy source (Fujii et al. 2000; Ushida et al. 2003). Yuan et al. (2004) suggested that NP and NP1EO were degraded by *Pseudomonas* sp. strain JC1 isolated from acclimated river sediments, but they did not note the degradation of NP2EO at all. Thus, no aerobic degradation of NP2EO to NP1EO and NP has been reported.

To confirm the biodegradability of these short EO chain-NPs such as NP1EO and NP2EO and to verify the metabolic pathway for them under aerobic conditions, $NPEO_{av2.0}$ was used as a sole carbon source in the screening of aerobic bacteria degrading short EO chain-NPs. Here, we report the isolation of $NPEO_{av2.0}$-utilizing axenic culture and propose a metabolic pathway for a short EO chain-NP to NP by aerobic bacteria. This is the first report on aerobic degradation of NP2EO to NP1EO and NP.

Materials and methods

Materials

$NPEO_{av2.0}$ was kindly provided by NIKKO Chemicals, Tokyo, Japan, as a mixture of unknown impurity (6.9%), NP1EO (32.6%), NP2EO (39.0%), nonylphenol triethoxylate (NP3EO, 16.2%), and nonylphenol tetraethoxylate (NP4EO, 5.3%); the average number of ethoxy (EO) units was approximately 2.0. The mixture did not contain NP at all. Nonylphenol polyethoxylate (averaging 10 EO units: $NPEO_{av10}$) and Triton X-100 (octylphenol polyethoxylate: averaging 10 EO units) were products of Tokyo Kasei Kogyo, Tokyo, Japan. *p*-Nonylphenol (NP), which is a mixture of branched nonylphenol isomers, was purchased from Nacalai Tesque, Kyoto, Japan. *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS), used for trimethylsilylation, were purchased from Supelco, Bellefonte, PA, USA. All other chemicals used in this study were of the highest grade available, and solvents for high-performance liquid chromatography (HPLC) were of HPLC grade.

Screening of $NPEOav_{2.0}$-utilizing bacteria

Activated sludge samples obtained from the Kurashiki Environmental Center (Kurashiki, Japan) were used as microbial sources. The sewage sludge samples were acclimated to $NPEO_{av2.0}$ (through step-by-step increases in concentration from 0.01 to 0.05%) following the method described previously (Kawai et al. 1977). One milliliter of the finally acclimated sludge suspension was added to 50 ml of basal mineral medium (BMM) supplemented with 0.1% $NPEO_{av2.0}$ in a 500-ml Erlenmeyer flask. BMM (pH 7.0) was prepared in distilled water, which included K_2HPO_4, 3.5 g /L; NaH_2PO_4, 2.2 g/L; $(NH_4)_2SO_4$, 1.0 g/L; $MgSO_4{\cdot}7H_2O$, 0.15 g/L; and yeast extract, 0.2 g/L. After shaking at 28°C for 1 week, 1 ml of the culture was transferred to a new BMM–$NPEO_{av2.0}$ flask. This procedure was performed a total of eight times. Finally, 100 μl of culture broth diluted appropriately was spread on BMM–$NPEO_{av2.0}$ agar plates to isolate single pure colonies. To prepare BMM–$NPEO_{av2.0}$ agar, BMM supplemented with $NPEO_{av2.0}$ was subjected to sonic oscillation with a UD-200 ultrasonic disrupter (Tomy, Tokyo, Japan) to disperse $NPEO_{av2.0}$ uniformly prior to autoclaving and solidified with 2% agar. Growths of isolated colonies were compared on BMM supplemented with 0.1% $NPEO_{av2.0}$, and selected strains were subjected for further study.

Identification of bacterial strains

Morphological features of the isolates were studied using a confocal laser scanning microscope (LSM 510, Carl Zeiss, Oberkochen, Germany). Gram-staining was carried out according to the method of Murray et al. (1994). Culture characteristics were observed according to Smibert and Krieg (1994). The 16S rDNA fragments of isolated strains were amplified by using universal oligonucleotide primers described by Kane et al. (1993). Sequences of the amplified fragments were analyzed with an ABI PRISM 377-18 DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA). The basic local alignment search tool (BLAST) program (http://blast.genome.jp/) was used to analyze homologies (Thompson et al. 1994). Detailed physiological and biochemical characteristics were identified by the National Collection of Industrial and Marine Bacteria (NCIMB) Japan (Shizuoka, Japan).

Determination of growth and degradation rates

Isolated bacteria were grown on BMM–$NPEO_{av2.0}$ under the same conditions as described above. The growth was determined by measuring the optical density at 610 nm with a UV-1600 spectrophotometer (Shimadzu, Kyoto, Japan). $NPEO_{av2.0}$ or NP was extracted from cultures of degraders and nondegraders (used as a control) as follows. Cultures (50 ml each) were mixed with equal volume of methanol and shaken vigorously to dissolve $NPEO_{av2.0}$ or NP, which was centrifuged at 8,000×*g* for 10 min to remove cells. The supernatant obtained was evaporated in vacuo to about 50 ml. The concentrated supernatant was extracted twice with 15 ml dichloromethane, and then the extracts were dehydrated with Na_2SO_4, filtered, and dried. The dried samples were dissolved in a 2-ml solvent mixture containing 98% ethyl acetate and 2% ethanol, and then subjected to HPLC. The HPLC system (Tosoh, Tokyo, Japan) consisted of a PX8020 solvent programmer, a CCPM multipump, an SD-8020 online degasser, an AS-8020 autosampler, a CO-8020 column oven, and a PD-8020 multiwavelength spectrophotometric detector, all of which were controlled by the PD-8020 analysis program (version 4.31). NP or $NPEO_{av2.0}$ was eluted with a mixture of 98%

ethyl acetate and 2% ethanol at a flow rate of 1.0 ml/min on a prepacked silica gel column (Cosmosil 5SL-II; Nacalai Tesque, Kyoto, Japan; 4.6 mm i.d. × 250 mm). Peaks were detected by measuring absorption at 281 nm (Liu et al. 2005). The degradation rate was calculated according to the equation described below. The recoveries of $NPEO_{av2.0}$ and NP from control cultures were more than 90%. The values were expressed on a mean value for three repeated experiments performed independently.

Degradation rate (%)={1-[the peak area of NP*n*EO (*n*=1–4) after incubation]/[the peak area of NP*n*EO (*n*=1–4) before incubation]}×100.

Preparation of NP1EO and NP2EO

NP1EO and NP2EO were isolated from $NPEO_{av2.0}$ by chromatography on a silica gel column. A 2.62-cm-i.d. × 3.6-cm silica gel column was packed with a mixture of Wakogel C-200 (75~150 μm) and Wakogel C100 (150~425 μm) (50:50) (Wako Pure Chemical Industries, Osaka, Japan). After applying an appropriate amount of $NPEO_{av2.0}$, the column was eluted with chloroform–hexane (35:65), where NP1EO–NP4EO was eluted separately. Each peak was further purified on the same column. The average purities of NP1EO and NP2EO were confirmed by HPLC to be about 99% and 97%, respectively.

Reaction of resting cells with NP1EO and NP2EO

Strains AS08 and AS90 were grown under optimized conditions (described in Results). Cells at the early stationary phase were collected by centrifugation at 8,000×*g* for 20 min and washed twice with 0.05 M Tris–HCl buffer (pH 8.0); these cells served as resting cells suspended in the same buffer at approximately 1.0 of absorbance at OD_{610}. Resting cells were incubated at 28°C under shaking with either 50 ppm NP1EO or NP2EO. In 3 days, the reaction mixtures were extracted twice, each time using a half volume of dichloromethane under neutral conditions and pH 2.0. The extracts were dehydrated with Na_2SO_4 and dried up, and then were subjected to HPLC and gas chromatography–mass spectrometry (GC–MS), with reference to Ahel et al. (1987).

GC–MS analysis

Dried samples were dissolved in ethyl acetate and trimethylsilylated by a mixture of BSTFA and TMCS, according to the instructions of the manufacturer of the GC–MS system. The GC–MS system (HP5980 Series 2 for GC and HP5971 for MS, Hewlett-Packard, Palo Alto, CA, USA) was equipped with a fused silica capillary column (HP-ultra2, 0.2 mm i.d. × 50 m, Hewlett-Packard). Helium was used as the carrier gas, and the flow rate was 1 ml/min. A 1-μl sample was injected splitless at 270°C, and after 30 s, a GC temperature program was started as follows: isothermal at 60°C for 2 min, increased to 300°C at 20°C/min, and then maintained at 300°C for 13 min. The mass spectra were measured under the following conditions: ion source temperature of 200°C, electron energy of 70 eV, scan mode MS/MS, and scan rate of 0.76 s. Compounds were identified with reference to previous papers (Ahel et al. 1987; Planas et al. 2002; Maki et al. 1994; John and White 1998).

Preparation of cell-free extracts and assay of enzymatic activity

Ensifer sp. strain AS08 was grown under optimized conditions (described in Results). Cells at the early stationary were collected by centrifugation at 8,000×*g* for 20 min, washed twice with cold 0.05 M Tris–HCl buffer (pH 8.0), and suspended in an appropriate amount of the same buffer. The suspended cells were disrupted by sonication at 200 W and 20 kHz under 0°C for 2 min repeatedly for eight times and then were centrifuged at 10,000×*g* for 30 min. The resultant supernatant was used as a cell-free extract after filtration with a 0.2-μm-pore-size filter. $NPEO_{av2.0}$ dehydrogenase activity was measured by the increase in absorbance at 570 nm of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT). A 1-ml reaction mixture included an appropriate amount of cell-free extract, 0.1 mM potassium cyanide, 0.1 mM MTT, 0.1 mM phenazine methosulfate (PMS), and 5 mM substrate (solved in *n*-dodecyl maltoside at a final concentration of 0.2%) in 0.1 M Tris–HCl buffer (pH 8.0) with and without 0.1 mM nicotinamide adenine dinucleotide (NAD). As a control, a reaction mixture containing no substrate was used. One unit of enzymatic activity was defined as the amount of enzyme that catalyzed the reduction of 1 ìmole of MTT per min (ε_{570}=6,410 $M^{-1}cm^{-1}$) under the standard assay conditions. The specific activity was defined as the units of enzyme contained per milligram of protein. The amount of protein was determined using the Bio-Rad Protein Assay kit (Bio-Rad, Burlingame, CA, USA) with bovine serum albumin as the standard. Formations of aldehydic compounds as reaction products were measured by measuring the absorbance at 580 nm of the 2,4-dinitrophenylhydrazone of the products with that of glycolaldehyde as the standard (Kawai et al. 1983).

Results

Screening of $NPEO_{av2.0}$-assimilating bacteria

After enrichment cultures on $NPEO_{av2.0}$ were performed as described in the "Materials and methods" section, the final enrichment culture was diluted to 10^{-8}- and 10^{-9}-fold, and 100-μl portions of each dilution were spread on BMM–$NPEO_{av2.0}$ agar plates. After 3 to 4 days' cultivation at 28°C, eight different bacterial colonies were recognized and purified. Among them, two strains (AS08 and AS90) were finally selected based on their growth on $NPEO_{av2.0}$

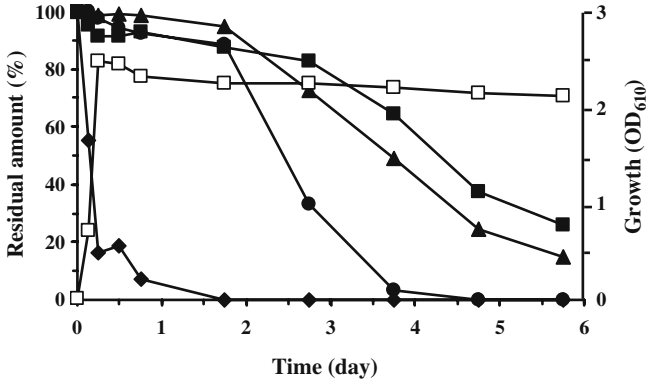

Fig. 1 Time course of cell growth and degradation of $NP_{av2.0}$ by *Ensifer* sp. strain AS08. $NP_{av2.0}$ was added at 0.02% to BMM. Growth and residual amounts of each compound were measured as described in "Materials and methods". Symbols used: *open square* growth, *closed square* NP1EO, *closed triangle* NP2EO, *closed circle* NP3EO, and *closed diamond* NP4EO

and related compounds (NP, $NPEO_{av10}$, and Triton X-100) in BMM, as described in the "Materials and methods" section. Two strains grew on $NPEO_{av2.0}$ and NP, but neither of the strains grew on $NPEO_{av10}$, nor on Triton X-100.

Identification of bacteria

Strain AS08 was a pair of rods that were both motile and gram-negative. Strain AS90 was a single rod, motile, and gram-negative. Partial 16S rDNA sequences were determined following the methods described in the "Materials and methods" section and deposited in GenBank under accession numbers AY662684 for strain AS08 and AY 622320 for strain AS90. The sequences of strains AS08 and AS90 showed the highest homology with genera *Ensifer* and *Pseudomonas*, respectively, by the BLAST program. Biochemical and physiological characterizations of the strains were practiced by NCIMB (data not shown), the results of which corresponded to those of the genera described above. Therefore, strains AS08 and AS90 were identified as *Ensifer* sp. strain AS08 and *Pseudomonas* sp. strain AS90, respectively, which have the accession numbers FERM P-20570 and P-20571, respectively, in the International Patent Organism Depositary (Tsukuba, Japan).

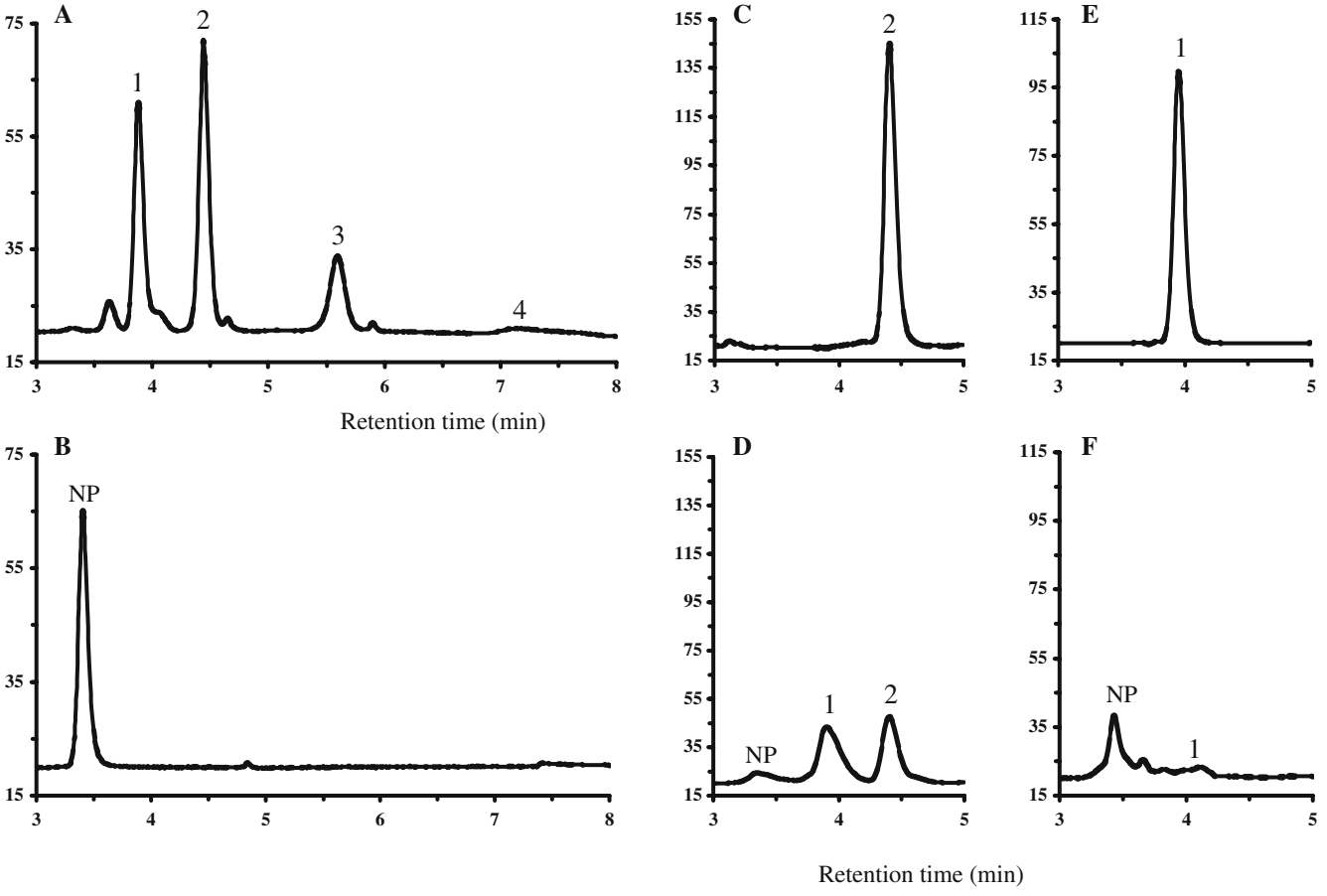

Fig. 2 Degradation of NP2EO and NP1EO by resting cells of *Ensifer* sp. strain AS08. Reaction was performed as described in "Materials and methods." **a** $NPEO_{av2.0}$, **b** NP, **c** NP2EO, **d** metabolites from NP2EO, **e** NP1EO, and **f** metabolites from NP1EO. *Peaks 1–4* included in $NPEO_{av2.0}$ correspond to NP1EO–NP4EO, respectively

Degradation of NPEOav2.0 by *Ensifer* sp. strain AS08 and *Pseudomonas* sp. AS90

The growth of two isolated strains on $NPEO_{av2.0}$ was poor (less than 0.3 by absorbance at 600 nm against a control culture without $NPEO_{av2.0}$), but degradation of $NPEO_{av2.0}$ happened gradually after reaching the maximum growth, indicating that $NPEO_{av2.0}$ was degradable, but not a good carbon source for assimilation. Addition of glucose or NP as a carbon source and ammonium salts or tryptone as a nitrogen source promoted the growth of both strains, but degradation of $NPEO_{av2.0}$ (0.02%, supplemented to BMM) was not improved at all. On the other hand, the presence of yeast extract (0.02–0.5%) promoted the growth and degradation remarkably, both of which were increased with increased concentrations of yeast extract. Growth reached the maximum in 10 h (Fig. 1). As the amount of NP4EO was only 5.3%, it rapidly degraded on a reverse parallel with growth and disappeared in 2 days. Degradation of NP3EO–NP1EO, however, was very slight in 2 days for both NP3EO and NP2EO and 3 days for NP1EO, and then rapidly increased, suggesting the induction of degradation ability. Degradation rate decreased with shorter ethoxy chain-NPs. The growth curve and degradation rate by *Pseudomonas* sp. strain AS90 was similar to those of strain AS08. As further cultivation for more than

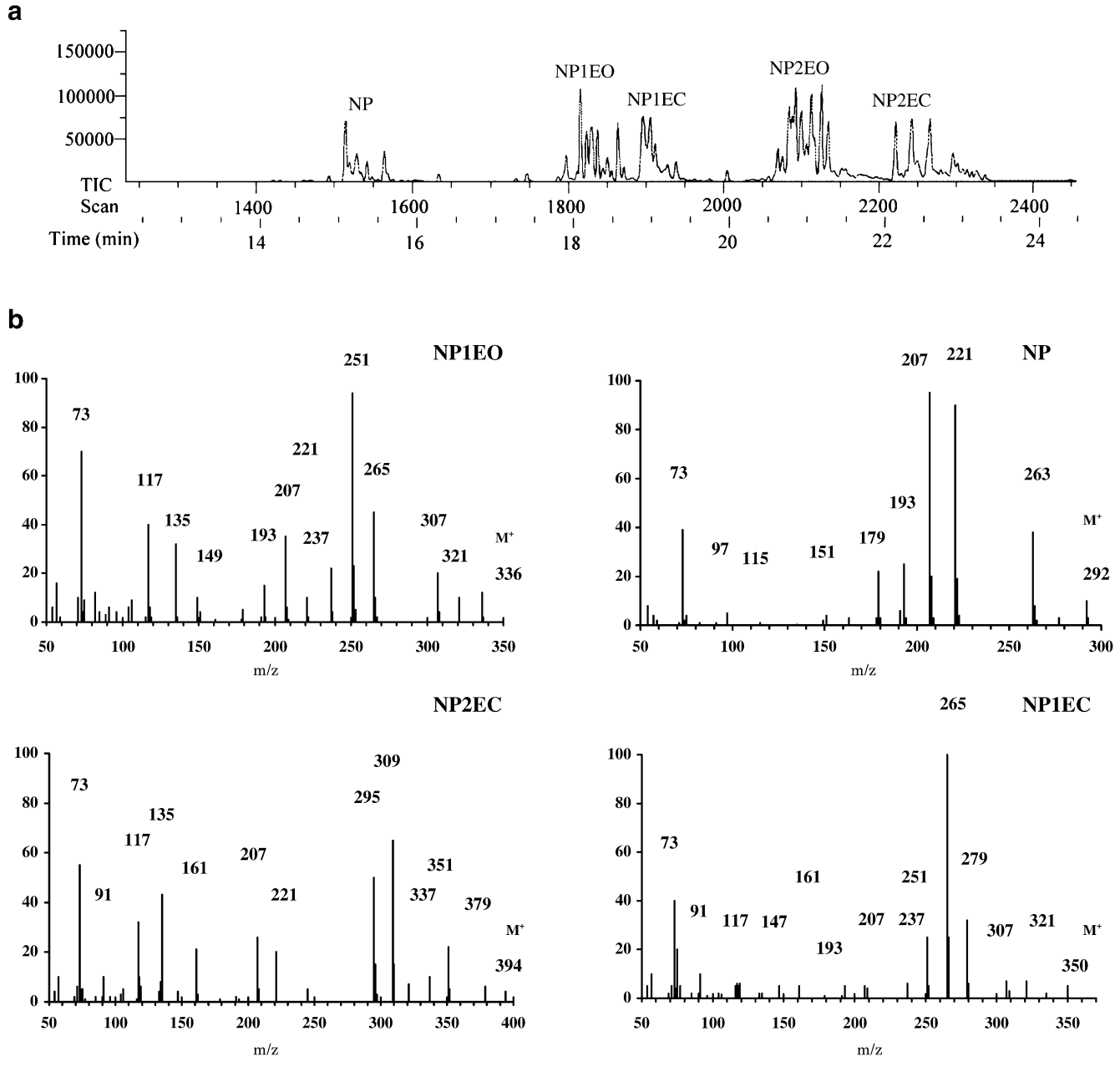

Fig. 3 Total ion chromatography (TIC) and mass spectra of metabolites from NP2EO and NP1EO **a** TIC. **b** Mass spectra. M^+ indicates the molecular mass of trimethylsilylated compounds

10 days showed better degradation rate by strain AS08 than by strain AS90, the strain AS08 was selected for further experiments. Both strains degraded NP by over 90% in 10 days when grown on BSM-NP (0.02%) supplemented with 0.5% yeast extract.

Identification of metabolites from NP2EO and NP1EO

The resting cells of *Ensifer* sp. strain AS08 and *Pseudomonas* sp. strain AS90 were incubated with NP1EO and NP2EO, respectively. In 3 days, the extracts with dichloromethane under neutral conditions were analyzed by HPLC to detect nonpolar compounds such as NP, NP1EO, and NP2EO. The acidic extracts with dichloromethane were trimethylsilylated, as described in the "Materials and methods" section, and subjected to GC–MS. The two strains showed similar degradation patterns and produced similar metabolites. NP2EO (Fig. 2c) was decreased, and two new peaks appeared on HPLC (Fig. 2d), the retention times of which corresponded to NP1EO and NP. NP1EO (Fig. 2e) was also consumed, and a peak corresponding to NP appeared on HPLC analysis, as shown in Fig. 2f. GC–MS analysis of acidic extracts from a reaction mixture with NP2EO showed that NP2EC, NP1EO, NP1EC, and NP were produced. On the other hand, NP1EC and NP were produced from NP1EO. Total ion chromatography indicated many peaks (derived from many isomers of NP) for each compound, as shown in Fig. 3a. The representative mass spectra of each component among various spectra obtained are shown in Fig. 3b. The mass spectrogram of NP was similar to that of authentic NP. These results from Figs. 2 and 3 suggest that oxidation at a terminal alcohol group is the first step in the degradation of NP2EO or NP1EO, followed by depolymerization of the EO chain until this chain is completely removed to yield NP.

Existence of alcohol dehydrogenase acting on NPEOav2.0

The reduction of several electron acceptors was examined with cell-free extracts prepared from *Ensifer* strain sp. AS08 grown on $NPEO_{av2.0}$ as described in the "Materials and methods" section. $NPEO_{av2.0}$ dehydrogenase activity was found with MTT coupled with PMS. Activity was low only in the presence of MTT plus PMS, but increased by two times or more by further addition of NAD. The enzyme was active toward NPEOs, $NPEO_{av2.0}$, NP2EO, NP1EO, and PEG 400, but not on tetraethylene glycol–diethylene glycol, as shown in Table 1. Formation of aldehydic compounds in a reaction mixture of cell-free extracts with each substrate was confirmed by the method described in the "Materials and methods" section. A reaction mixture of cell-free extract without substrate was used as a control. The amount of aldehydes produced approximately corresponded to the enzyme activity. No activity was found with cell-free extract prepared from cells grown on nutrient broth, suggesting that $NPEO_{av2.0}$ induced enzymatic activity.

Table 1 Enzymatic activity toward $NPEO_{av2.0}$ and related compounds

Substrate	Activity (mU/mg protein)		Aldehyde formed (nmole/mg protein/min)
	-NAD	+NAD	
$NPEO_{av2.0}$	11	25	23
NP2EO	9.0	37	33
NP1EO	12	28	29
NPEOs	12	34	16
PEG 400	nd	12	13
Tetraethylene glycol	nd	nd	nd
Triethylene glycol	nd	nd	nd
Diethylene glycol	nd	nd	nd

The formation of aldehydes was measured in the presence of NAD as described in Materials and methods. nd, not detected

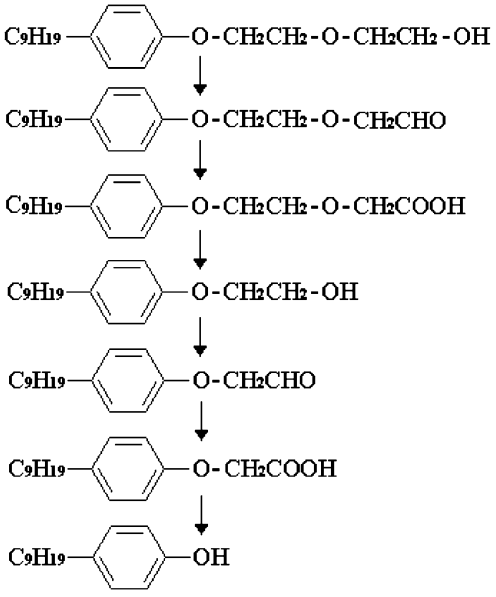

Fig. 4 Proposed aerobic metabolic pathway from NP2EO to NP

Discussion

To learn the biodegradability of each short EO chain-NP (especially NP2EO and NP1EO) under aerobic conditions and to elucidate their metabolic pathways, $NPEO_{av2.0}$-assimilating bacteria were screened from sewage sludge by enrichment culture techniques. Two aerobic bacterial isolates able to grow on $NPEO_{av2.0}$ were isolated and identified as *Ensifer* sp. strain AS08 and *Pseudomonas* sp. strain AS90. This is the first report of aerobic bacteria able to assimilate all of NP4EO ~ NP1EO, especially estrogenic NP2EO and NP1EO. As both strains also assimilate NP, all of the endocrine disrupters (NP2EO, NP1EO, and NP) derived from NPEOs are removed by using either strain.

The growth and degradation rates of $NPEO_{av2.0}$ by strains AS08 and AS90 were remarkably improved by increasing the amount of yeast extract from 0.02% to 0.5%. Hayashi et al. (2005) showed biodegradation of NPEOs in

the presence of organic matter, forming the corresponding NPEOs-carboxylates by the oxidation of the terminal alcohol group as temporary intermediates under a modified OECD 301E biodegradation test protocol. Without organic matter, NP2EO and NP3EO were predominant metabolites of long-chain precursors and were retained in the substrate for a long time. Several other researchers have reported that increasing the amount of yeast extract enhanced the transformation of phenanthrene in soil (Yuan et al. 2000) and that of NP and NP1EO in river sediment (Fujii et al. 2000). Ushida et al. also described that a novel NP-degrading *Sphingobium amiense* sp. nov. could degrade NP in the presence of an organic nutrient such as yeast extract, but could not use NP as a carbon source (Ushida et al. 2003). An appropriate addition of supplementary nutrients seems to be indispensable for promoting degradation; such supplementation corresponds to a biostimulation technique in the bioremediation of pollutants in situ.

Thus far, two mechanisms have been known to be involved in the transformation of NPEOs to NP2EO. Some NPEOs degraders, such as *Pseudomonas* sp. strain 14-1 (Maeda and Mikami 1988) and *Pseudomonas* sp. strain TR01, degraded NPEOs to NP2EO through oxidation of the EO chain to produce carboxylates as intermediate metabolites. On the other hand, John and White (1998) suggested that *Pseudomonas putida* transformed NPEOs to NP2EO aerobically or anaerobically through the hydroxyl shift from the terminal carbon to the adjacent carbon to produce a hemiacetal, where no carboxylates were produced, followed by the split to acetaldehyde and the resultant EO chain-NP that is one EO unit shorter. The pathway was first proposed for anaerobic metabolism of PEG (Frings et al. 1992). Sato et al. (2003) have also suggested oxidative biodegradation for uniform octylphenol polyethoxylate (OP*n*EO) isolated from Triton X-100. A series of OP*n*EO molecules (n=2–8) and their corresponding carboxylic products were observed in the culture supernatant of *P. putida* S-5. OP3EO and OP2EO were accumulated in the culture supernatant to show that these compounds might be dead-end products. The transformation of NP2EO further to NP1EO has not been noted so far. Therefore, their degradation necessitates another microorganism such as *Ensifer* sp. strain AS08 or *Pseudomonas* sp. strain AS90.

The present study clearly suggests that aerobic bacteria first attacked a terminal alcohol group of the EO chain in short EO chain-NP by MTT-dependent short EO chain-NP dehydrogenase (sEONP-DH). Maki et al. (1994) found that *Pseudomonas* sp. strain TR01 metabolized the EO chain only when the chain linked to bulky hydrophobic groups such as alcohol and alkyl phenol, but its metabolic enzyme has not been characterized yet. Whether or not sEONP-DH of *Ensifer* sp. strain AS08 is similar to PEG dehydrogenase (PEG-DH) (Sugimoto et al. 2001) still awaits further studies. As enhancement of PEG-DH activity by NAD has not been reported and shorter EO chain-NP is far more hydrophobic than PEG, this might suggest a different dehydrogenase. The existence of sEONP-DH together with aldehydic products and the production of NP2EC and NP1EC as metabolites from NP2EO and NP1EO, respectively, revealed that the oxidation of a terminal alcohol group was the primary step in aerobic degradation of short EO chain-NP. Depolymerization must follow to yield an EO chain-NP that is shorter by one EO unit until NP is finally obtained. As no difference was found between metabolites produced by strain AS08 and those produced by strain AS90, these strains must share a common aerobic metabolism of short EO chain-NPs. As shown in Fig. 4, this two-step process is repeated until NP is produced.

Acknowledgements We are grateful to Dr. M. Hatta, Okayama University of Science, and to Mr. Y. Haba for their help with the GC–MS measurement. This work was partly supported by the Research Grant for Encouragement of Studies to F. K. and X. L. from the Graduate School of Natural Science and Technology, Okayama University. We appreciate the review of the manuscript prior to submission by KN International.

References

Ahel M, Conrad T, Giger W (1987) Persistent organic chemicals in sewage effluents. 3. Determinations of nonylphenoxy carboxylic acids by high-resolution gas chromatography/mass spectrometry and high-performance liquid chromatography. Environ Sci Technol 21:697–703

Ejlertsson J, Nilsson ML, Kylin H, Bergman A, Karlson I, Oquist M, Svensson BH (1999) Anaerobic degradation of nonylphenol mono- and diethoxylates in digestor sludge, landfill municipal solid waste and landfill sludge. Environ Sci Technol 33: 301–306

Frings J, Schramm Z, Schink B (1992) Enzymes involved in anaerobic polyethylene glycol degradation by *Pelobacter venetianus* and *Bacteroides* strain PG1. Appl Environ Microbiol 58:2164–2167

Fujii K, Urano N, Ushio H, Satomi M, Iida H, Ushio-Sata N, Kimura S (2000) Profile of a nonylphenol-degrading microflora and its potential for bioremedial applications. J Biochem 128:909–916

Giger W, Brunner PH, Schaffner C (1984) 4-Nonylphenol in sewage sludge: accumulation of toxic metabolites from nonionic surfactants. Science 225:623–625

Hayashi S, Saito S, Kim J-H, Nishimura O, Sudo R (2005) Aerobic biodegradation behavior of nonylphenol polyethoxylates and their metabolites in the presence of organic matter. Environ Sci Technol 39:5626–5633

Jobling R, Sumpter JP (1993) Detergent components in sewage effluent are weakly estrogenic to fish: an in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquat Toxicol 27:361–372

John DM, White GF (1998) Mechanism for biotransformation of nonylphenol polyethoxylates to xenoestrogens in *Pseudomonas putida*. J Bacteriol 180:4332–4338

Jonkers N, Knepper TP, de Voogt P (2001) Aerobic biodegradation studies of nonylphenol ethoxylates in river water using liquid chromatography–electrospray tandem mass spectrometry. Environ Sci Technol 35:335–340

Jonkers N, Laane RW, de Voogt P (2003) Fate of nonylphenol ethoxylates and their metabolites in two Dutch estuaries: evidence of biodegradation in the field. Environ Sci Technol 37:321–327

Kane MD, Poulsen LK, Stahl DA (1993) Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences. Appl Environ Microbiol 59:682–686

Kawai F, Fukaya M, Tani Y, Ogata K (1977) Identification of polyethylene glycols (PEGs)-assimilable bacteria and culture characteristics of PEG 6000 degradation by a mixed culture. J Ferment Technol 55:429–435

Kawai F, Kimura T, Tani Y, Yamada H, Ueno T, Fukami H (1983) Identification of reaction products of polyethylene glycol dehydrogenase. Agric Biol Chem 47:1669–1671

Kvestak R, Ahel M (1994) Occurrence of toxic metabolites from nonionic surfactants in the Krka River estuary. Ecotoxicol Environ Saf 28:25–34

Liu X, Tani A, Kawai F (2005) Determination of nonylphenol and short chain nonylphenol polyethoxylates by normal phase high performance liquid chromatography. Chin J Anal Chem 33:1189–1191

Maeda S, Mikami I (1988) Degradation of the non-ionic surfactant (polyoxyethylene-*p*-nonylphenyl ether) by *Pseudomonas* species. J Water Wastes 30:1056–1063

Maki H, Masuda N, Fujiwara Y, Ike M, Fujita M (1994) Degradation of alkylphenol ethoxylates by *Pseudomonas* sp. strain TR01. Appl Environ Microbiol 60:2265–2271

Montgomery-Brown J, Drewes JE, Fox P, Reinhard M (2003) Behavior of alkylphenol polyethoxylate metabolites during soil aquifer treatment. Water Res 37:3672–3681

Murray ERB, Doetsch RN, Robinow CF (1994) Determinative and cytological light microscopy. In: Gerhardt P, Murray ERB, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. ASM, Washington, DC, pp 31–32

Patoczka J, Pulliam GW (1990) Biodegradation and secondary effluent toxicity of ethoxylated surfactants. Water Res 24: 965–972

Planas C, Guadayol JM, Droguest M, Escals A, Rivera J, Caixach J (2002) Degradation of polyethoxylated nonylphenols in a sewage treatment plant. Quantitative analysis by isotopic dilution-HRGC/MS. Water Res 36:982–988

Sato H, Shibata A, Wang Y, Yoshikawa H, Tamura H (2003) Characterization of biodegradation intermediates of nonionic surfactants by MALDI-MS. 2. Oxidative biodegradation profiles of uniform octylphenol polyethoxylate in ^{18}O-labeled water. Biomacromolecules 4:46–51

Smibert RM, Krieg NR (1994) Phenotypic characterization. In: Gerhardt P, Murray, ERB, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. ASM, Washington, DC, pp 615–640

Soto AM, Justicia H, Wray JW, Sonnenschein C (1991) *p*-Nonylphenol: an estrogenic xenobiotic released from modified polystyrene. Environ Health Persp 92:167–173

Sugimoto M, Tanabe M, Hataya M, Enokibara S, Duine JA, Kawai F (2001) The first step in polyethylene glycol degradation by sphingomonads proceeds *via* a flavoprotein alcohol dehydrogenase containing flavin adenine dinucleotide. J Bacteriol 183:6694–6698

Thompson JD, Higgins DG, Gibson TJ (1994) Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Ushida Y, Takahara Y, Ohta H (2003) *Sphingobium amiense* sp. nov., a novel nonylphenol-degrading bacterium isolated from a river sediment. Inter J Syst Evol Microbiol 53:2045–2048

Wintgens T, Gallenkemper M, Melin T (2003) Occurrence and removal of endocrine disrupters in landfill leachate treatment plants. Water Sci Technol 48:127–134

Yuan SY, Chang JS, Yen J, Chang BV (2000) Biodegradation of phenanthrene in river sediment. Chemosphere 43:273–278

Yuan SY, Yu CH, Chang BV (2004) Biodegradation of nonylphenol in river sediment. Environ Pollut 127:425–430