Short Communication

Characteristics of Nonylphenol Polyethoxylate-Degrading Bacteria Isolated from Coastal Sediments

TOSHIMICHI MAEDA¹*, KAZUhide HAYAKAWA¹, MU YOU¹, MAKOTO SASAKI¹, YASUHIRO YAMAJI¹,
MANABU FURUSHITA¹ and TSUNEO SHIBA¹

¹ Department of Food Science and Technology, National Fisheries University, 2–7–1 Nagata-Honmachi,
Shimonoseki, Yamaguchi 759–6595, Japan

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Twenty-six bacterial strains, which degrade nonylphenol (and octylphenol) polyethoxylates and accumulate mono-, di-, and/or tri-ethoxylated homologues, were isolated from coastal sea sediments of Japan. According to restriction-fragment polymorphisms of the PCR-amplified 16S rRNA gene, the strains were divided into 7 genotypes. Phylogenetic analyses of the 16S rRNA gene indicated that the isolates were included in *Gammaproteobacteria*, and distinct from those of nonylphenol polyethoxylate-degrading bacteria isolated from active sludge and paddy fields.

Key words: nonylphenol polyethoxylates, octylphenol polyethoxylates, degradation, coastal sediment

Nonylphenol polyethoxylates (NPEOn; *n* designates the number of ethylene glycol units) are non-ionic surfactants mainly utilized in industrial processes. The surfactants are comprised of a phenol nucleus, a variety of hydrophobic nonyl moieties, and a hydrophilic polyethylene glycol chain ether-linked at the phenolic oxygen. Surfactants produced commercially are a mixture of the compounds with ethoxylate chains ranging in length from 1 to typically 18 ethylene glycol units.

NPEOn discharged into the natural environments are efficiently degraded and transformed into recalcitrant products, nonylphenol (NP), nonylphenol mono- or di-ethoxylate (NPEO₁ or NPEO₂), and nonylphenol mono- or di-ethoxy carboxylate (NPEC₁ or NPEC₂)^{4,7,22}. Since these products show estrogenic activities^{9,23}, the degradation process in the environments is of public concern. Although NPEOn eventually flow into seawater environments and accumulate in coastal sediments^{3,15}, only a few marine octylphenol

polyethoxylate (OPEOn)-degrading bacteria have been reported¹⁶, in contrast to the many terrestrial NPEOn- and OPEOn-degrading bacteria reported^{2,8,10,14,17}. Hence, this paper describes the phylogenetic and phenotypic characteristics of marine NPEOn-degrading bacteria isolated from coastal environments.

The bacteria were isolated by inoculating ca. 0.1 g of coastal sediments into 10 ml of a sterilized seawater medium, SM-N. The medium was comprised of natural seawater filtrated through a baked glass fiber filter (GF/F, Whatman Co., Middlesex, UK) and 0.1% Triton N101 (a mixture of NPEOn with 1 to 8 ethylene glycol units, Sigma-Aldrich Co., Steinheim, Germany), 9 mM NH₄Cl and 0.03 mM Na₂HPO₄; pH 7.5. The suspension was shaken at 30°C for 2 to 4 weeks. NPEOn were extracted by adding an equal volume of chloroform to 0.5 ml of the suspension, and then each homologue (NPEOn with a different number of ethylene glycol units) was quantified by high performance liquid chromatography (HPLC)^{1,21}. The number of ethylene glycol units in the homologues fractionated by HPLC was determined by gas chromatography/mass spectrometry (GC/

* Corresponding author; E-mail: toshima@fish-u.ac.jp, Tel: +81–832–86–5111, Fax: +81–832–86–7434

MS)¹⁰. When degradation of NPEOn was observed, the suspension was spread on a PPES-II agar medium²⁴) containing 0.1% Triton N101. Strains were isolated from colonies with a turbid halo and then inoculated into an artificial seawater medium, ASM-N, that contained 0.1% Triton N101, 0.3 M NaCl, 0.02 M MgSO₄, 0.01 M KCl, 0.01 M CaCl₂, 5 mM KNO₃, 9 mM NH₄Cl, 0.03 mM Na₂HPO₄, 0.01% yeast extract (Bacto, Difco Laboratories, Livonia, USA), 74 nM vitamin B₁₂, 0.001% ferric citrate, 0.01% trace metal solution, and 50 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonate (HEPES); pH 7.5. If the turbidity of the cultures at 600 nm was more than 0.2 in the medium after 2 weeks at 30°C, the strains were identified as positive for NPEOn degrading activity. The turbidity in the medium without Triton N101 was less than 0.01.

16S rRNA genes (approximately 1,500 bp) of the strains were PCR-amplified as described previously¹³) and analyzed based on their restriction fragment length polymorphisms (RFLP)¹²) using 9 different 4-base-recognizing restriction enzymes, *Nde*II, *Taq*I, *Hae*III, *Hha*I, *Rsa*I, *Hap*II, *Aha*I, *Tru*9I and *Tsp*509I. The 16S rRNA gene was sequenced with a Dye terminator cycle sequencing kit and an Applied Biosystems 310 DNA sequencer. The GenBank/EMBL/DDBJ accession numbers for the sequences are AB212800 to AB212806. A phylogenetic tree was constructed by the neighbor-joining method, referring to the Ribosomal Database Project⁶) and GenBank databases, and using the software Clustal W (version 1.83)²⁵) and Kimura's two-parameter model.

Phenotypic characteristics were examined by conventional methods²⁰). Utilization of organic carbon was examined with ASM-N medium containing 0.1% of the organic carbon source in place of Triton N101. Utilization of OPEOn was examined with Triton X100 (a mixture of OPEOn with 1 to 18 ethylene glycol units, Sigma-Aldrich Co., Steinheim, Germany.).

In total, 26 NPEOn-degrading bacteria were isolated from the coastal sediment sampled at Shimonoseki, Kochi and Tokyo. According to the RFLP of the PCR-amplified 16S rRNA gene, the strains were divided into 7 genotypes: type A, strain NEP1; type B, strain NEP2; type C, strains NEP3, 7, 8, 10, 11, 12, 13, 14, 15; type D, strains NEP4, 5, 6, 9, 14; type E, strains NEP17, 18, 19, 20, 21, 22, 23; type F, strains NEP65, 66; and type G, strain NEP68. Strains of genotypes A, B, C, D and E were isolated from the coastal sediment sampled at Shimonoseki, whereas strains of genotypes F and G were isolated from the coastal sediment sampled at Kochi and Tokyo, respectively.

A phylogenetic analysis based on the 16S rRNA gene se-

quences (1,455–1,456 bp) showed that representative strains of all genotypes belonged in *Gammaproteobacteria* (Fig. 1). The similarity of the sequences between the 7 strains ranged from 89.1% (strain NEP2 vs. NEP65) to 99.5% (strain NEP1 vs. NEP65). Strain NEP2 (genotype B) was far separated from the others, and showed the highest similarity (95.4%) to a marine isolate, SCB11¹⁸). Strains NEP1, NEP3, NEP17 and NEP65, representative of genotypes A, C, E and F, respectively, made a small group with an arctic sea ice bacterium, ARK10038⁵), while strain NEP4 (genotype D) and NEP68 (genotype G) were slightly separated from them. The strains were far separated from terrestrial NPEOn-degrading bacteria isolated from activated sludge; strains BC16, 47, 55, 111 and 187 that belonged to *Aeromonas*, *Shewanella*, *Proteus* and *Acinetobacter*²). The strains were also far from *Pseudomonas putida* and *Burkholderia cepacia* which include NPEOn- and OPEOn-degrading bacterial strains isolated from sewage sludge and paddy field^{8,17}).

All of the strains tested in this study were Gram-negative, motile short rods, catalase-positive, oxidase-positive, required Na⁺ for growth, contained ubiquinone 8 as a major quinone, and grew at temperatures ranging from 20 to 40°C. The colonies were a white or cream color. All of the strains grew at pH 7 to 9, except for strain NEP2 which did not grow at pH 9. All of the strains utilized NPEOn, OPEOn, acetate, pyruvate, propionate, succinate, lactate and tetraethylene glycol, but not glycolate, monoethylene glycol, diethylene glycol, or polyethylene glycol 4000 as a sole carbon source. Polyethylene glycol 300 was utilized by all of the strains, except for strain NEP2.

Figure 2 shows a typical change in the composition of homologues of NPEOn during the degradation of Triton N101. The amount of homologues with 7 and more ethylene glycol units steadily diminished, while that of NPEO₅ and NPEO₆ temporarily increased and then decreased. Along with the decrease of the longer homologues, the shorter homologues, NPEO₂, NPEO₃ and NPEO₄, were accumulated. In all of the degradations, a decrease in absorbance specific to the benzene ring (at 270 nm) was not observed (data not shown). Hence the growth with NPEOn is dependent only on the degradative assimilation of the polyethoxylate chain. The temporal increase in NPEO_{5,6} suggested a serial removal of ethylene glycol units at the terminal ether bond as discussed by John and White¹⁰). The shorter homologues were found to accumulate in all of the strains and the profile of their accumulation varied with the genotype: strains of genotype D accumulated NPEO₂; genotype A and F, NPEO_{2,3}; genotype B, NPEO₂₋₄; genotype C, NPEO₂₋₆; genotype E and G,

Fig. 1. Phylogenetic distribution of NPEOn-degrading bacteria, based on the nucleotide sequence of the 16S rRNA gene. Strains BC16, BC47, BC55, BC111 and BC187 are NPEOn-degrading bacteria isolated from activated sludge²¹. The numbers at each branch are bootstrap values ($n=1000$). The tree was constructed by the neighbor-joining method. Evolutionary distance was calculated according to Kimura's two-parameter model. The tree root was determined by using the 16S rRNA gene of *Cytophaga hutchinsonii* (M58768) as an outgroup species. The GenBank accession numbers are shown in the parentheses.

NPEO₁₋₃. The accumulation of the shorter homologues has been reported during degradation in an estuary¹¹.

The serial removal of ethylene glycol units should yield only acetate, not mono ethylene glycol or glycolate that are the reported probable products in the liberation of the glycol unit^{10,26}, since acetate but neither mono ethylene glycol nor

glycolate was utilized as a growth substrate. Although terminal oxidation of the ethylene glycol unit is also a likely mechanism of liberation¹⁹, no carboxylate analogs of NPE-On were found in the GC/MS analysis in this experiment.

Fig. 2. Changes in composition of NPEOn homologues during the degradation of Triton N101 by strain NEP2. NPEO₇ and longer homologues are indicated as lines without symbols. Since the molar extinction coefficients were identical for all of the NPEOn homologues¹⁾, the molar amounts of each homologue were calculated from the HPLC peak areas derived from a standard solution of Triton N101.

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