Original Article

Application of matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry for the identification of alkylphenol polyethoxylate-degrading bacteria in the environment

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In order to clarify the usefulness of the *S10*-GERMS (*S10*-*spc*-*alpha* operon gene-encoded ribosomal protein mass spectrum) method for the discrimination of microbial isolates from the environment, the isolates with octylphenol polyethoxylate (OPEO_n)-degrading capability from several soils in Japan were classified into 4 biodegradation patterns based on different final metabolic toxicants. Some isolates were identified as genera *Chelatococcus* and *Mesorhizobium* which have never been reported as OPEO_n-degrading bacteria based on 16S rRNA gene sequences. The findings from this study demonstrate that the *S10*-GERMS method successfully discriminates the isolates at the strain level in the genus *Pseudomonas*. Moreover, this method is better than 16S rRNA gene sequence similarity because it precisely demonstrated that OPEO_n-degrading bacteria in the genera *Chelatococcus* and *Mesorhizobium* might be new species. The *S10*-GERMS method is suggested as a useful tool for the discrimination and monitoring of man-made chemical-degrading bacteria isolated from the environment. © Pesticide Science Society of Japan

Keywords: alkylphenol polyethoxylate-degrading bacteria, octylphenol polyethoxylate, biodegradation, *S10*-GERMS method, MALDI-TOF MS.

Electronic supplementary materials The online version of this article contains supplementary materials (Supplemental Figure S1, Supplemental Tables S1 and S2), which is available at <http://www.jstage.jst.go.jp/browse/jpestics/>.

Introduction

Since pesticides that are discharged into the non-target environment through surface water and/or groundwater transform into their biodegraded products (metabolic toxicants), and may exert certain chemical selective pressure on wildlife, including animals, plants, and microorganisms the establishment of their life-cycle impact assessment in the environment is becoming more important in an environmentally conscious material cycle society. Nonionic surfactant octylphenol polyethoxylates (OPEO_n), which are one of the alkylphenol polyethoxylates (APEO_n), are easily degraded to octylphenols (OP), octylphenol monoethoxylate (OPEO₁), octylphenol diethoxylate (OPEO₂), and the corresponding octylphenol carboxylates (OPEC_{1,2}) in the environment. In particular, accumulated OP, OPEO₁, and OPEO₂, with their relatively high hydrophobicity in the environment, act as estrogen agonists and androgen antagonists.^{1–3)} Accumulated

knowledge of APEO_n biodegradation has revealed that there are two biodegradation mechanisms: 1) exo-type shortening of the EO chain accompanied by either oxidation of the EO moiety (oxidative biodegradation)^{4–8)}; or 2) nonoxidative hydroxy shift.^{9,10)} Moreover, environmental elements, such as Mg²⁺, Ca²⁺, and Fe³⁺, significantly influence the final degradation metabolites.^{3,11)}

In a previous study, we demonstrated that bacteria that can degrade OPEO_n to estrogenic and antiandrogenic metabolites are ubiquitous in paddy fields in Japan.^{12,13)} Furthermore, the study of the dynamic relationship between the microbial diversity and biodegradation capacity of OPEO_n using enrichment cultures of various sediments from Iwata River in Japan revealed that OPEO_n-degrading bacteria are widely distributed at the sites of different types of human activity along Iwata River.¹⁴⁾ This implies that the quantity and quality of the resultant ecotoxicity depended on the metabolites in the environment. Therefore, it is important to identify degrading bacteria rapidly for the evaluation and monitoring of the degrading activity of man-made chemicals.

Currently, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is used for the

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identification of microorganisms, especially, in routine clinical microbiological diagnostics because of its accuracy, speed, and cost effectiveness. However, a complete and representative database is an essential requirement for the accurate identification of isolates by MALDI-TOF MS method which is based on a principle called bacterial fingerprinting. Therefore, a database in which bacterial species are mislabeled or with a low number of registered bacterial species can cause misidentification by MALDI-TOF MS. In environmental microorganisms, the number of isolated and characterized prokaryotic species is still relatively low; thus, it is difficult to apply the MALDI-TOF MS method based on bacterial fingerprinting to the identification of environmental microorganisms. Therefore, the *S10*-GERMS (*S10*-*spc-alpha* operon gene-encoded ribosomal protein mass spectrum) method using ribosomal proteins coded in the *S10*-*spc-alpha* operon as biomarkers was developed to achieve a rapid and simple bacterial discrimination and typing method and demonstrated the advantages of such a bioinformatics-based approach over bacterial fingerprinting.^{15–17)} The objective of this study is to clarify the usefulness of the *S10*-GERMS method for the discrimination of bacteria isolated from the environment. For that purpose, therefore, various OPEO_n-degrading bacteria were also isolated.

This study demonstrated that the *S10*-GERMS method is clearly more suitable than 16S rRNA gene sequence similarity for the rapid discrimination of OPEO_n-degrading bacteria isolated from the environment that were grouped on the basis of final metabolic toxicants due to their degrading activity.

Materials and Methods

1. Culture media

t-Octylphenol polyethoxylates (OPEO_n), which have the

commercial name Triton X-100 (TX-100), were purchased from Wako (Japan) and Aldrich Chemical Co. (USA). A liquid basal salt medium with 0.1% (w/v) of TX-100 as a sole carbon source, called the TX-A medium, was used for the biodegradation test and isolation of OPEO_n-degrading bacteria. The TX-A medium is described in a previous study.¹¹⁾ The TX-A medium with 100 mM sucrose, called the TX-sucrose medium, was used for bacteria that do not shorten the ethoxylate (EO) chain of OPEO_n.

2. Isolation of OPEO_n-utilizing bacteria

OPEO_n-degrading bacteria were isolated from several soils in Japan. The isolated bacteria and sampling sites are shown in Table 1. The isolation procedures of OPEO_n-degrading bacteria are the same as those used for others except for the medium.¹¹⁾ Briefly, 0.5 g soil was added to 5 mL of a TX-A medium in a test tube. After shaking at 30°C for 2 weeks, a 100-μL sample suspension solution was transferred to 5 mL of a newly prepared TX-A medium. This procedure was performed three times. Finally, a 100-μL enriched sample solution was spread on the basal salt medium agar plate with 1% (w/v) TX-100. After incubation at 30°C for 1 week, single colonies were selected and subjected to further studies.

3. Bacterial biodegradation test

The isolated bacteria were incubated in 5 mL TX-A or TX-sucrose media. After shaking at 30°C for 14 days, the culture medium was extracted with an equal volume of ethyl acetate with 25 mg/L *n*-eicosane, and the ethylacetate layer was analyzed by gas chromatography (GC) and MALDI-TOF MS. GC analysis of biodegradation products was performed under the conditions described previously.¹¹⁾ MALDI-TOF MS measurements of

Table 1. Bacterial isolates used in this study

Strain	Source of isolation	Biodegradation pattern	Assignment of bacteria	Proteobacteria	Homology (%)
S5	Paddy field, Fukuoka	3	<i>Pseudomonas putida</i>	γ	98.8
FMP1	Paddy field, Fukuoka	3	<i>Pseudomonas putida</i>	γ	99.2
EC31	Upland field, Aichi	4	<i>Pseudomonas putida</i>	γ	98.8
BSN03	Ueda river, Aichi	1	<i>Chelatococcus asaccharovorans</i>	α	97.1
BSN07	Ibi river, Mie	1	<i>Chelatococcus asaccharovorans</i>	α	97.1
BSN11	Nagara river, Gifu	1	<i>Sphingopyxis macrogoltabidus</i>	α	99.9
BSN20	Ueda river, Aichi	2	<i>Sphingopyxis terrae</i>	α	99.9
BSN22	Upland field, Gifu	1	<i>Sphingopyxis soli</i>	α	98.5
BSN24	Yamayoke river, Mie	1	<i>Chelatococcus asaccharovorans</i>	α	97.1
BSN48	Upland field, Aichi	1	<i>Sphingopyxis soli</i>	α	98.5
BSN51	Upland field, Saitama	1	<i>Sphingopyxis ginsengisoli</i>	α	99.4
BSN53	Upland field, Saitama	2	<i>Sphingopyxis soli</i>	α	99.6
BSN54	Upland field, Saitama	2	<i>Sphingopyxis macrogoltabidus</i>	α	99.2
BSN55	Iwata river, Mie	2	<i>Sphingobium cloacae</i>	α	98.0
BSN58	Upland field, Aichi	3	<i>Mesorhizobium thioangeticum</i>	α	98.5
BSN59	Iwata river, Mie	1	<i>Chelatococcus asaccharovorans</i>	α	97.1
BSN60	Iwata river, Mie	1	<i>Chelatococcus asaccharovorans</i>	α	97.1

Fig. 1. Biodegradation pattern of TX-100 by representative OPEO_n-degrading bacteria.

(a) GC analysis of biodegradation products by BSN07 (Pattern 1), (b) GC analysis of biodegradation products by BSN53 (Pattern 2), (c) GC analysis of biodegradation products by FMP1 (Pattern 3), (d) MALDI-TOF MS analysis of biodegradation products by EC31 (Pattern 4).

carboxylated products of OPEO_n were performed using a Voyager DE-PRO time-of-flight mass spectrometer (Applied Biosystems, USA). As the matrix for sample ionization, 5,10,15,20-tetrakis (pentafluorophenyl) porphyrin (F20TPP; MW 974.6; Sigma, USA) was used. About 2 mg F20TPP was dissolved in 1 mL ethyl acetate to make the matrix solution. Sodium iodide (NaI) as a cationization salt was dissolved in ethyl acetate. The sample solution (1 μ L) was mixed with the matrix solution (5 μ L) and NaI solution in a glass tube with an inner diameter of 4 mm. About 1 μ L of the sample/matrix mixture was spotted onto the MALDI target and dried in air. MALDI mass spectra in the range of m/z 300–1500 were observed in a positive linear mode by averaging 300 individual laser shots.

4. 16S rRNA gene and *S10* and *spc* operon sequencing

Chromosomal DNA was extracted from the bacteria as described previously.¹⁵⁾ The quantity and quality of the extracted DNA were estimated by measuring the UV absorption spectrum (BioSpec-mini, Shimadzu, Japan). 16S rRNA gene sequencing for the identification of isolates was performed under the conditions described previously.¹¹⁾ PCR amplification was performed using the general bacterial primers 27f and 1492r.¹⁸⁾ The Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov/blast/>) program was used for homology analysis. Phylogenetic tree construction and bootstrap analyses (100 replicates) were performed using the Mega3.1 program.¹⁹⁾

PCR amplification of *S10* and *spc* operons was performed using KOD containing dNTP at a concentration of 200 μ M, each of the primers at a concentration of 4 μ M, 100 ng template DNA, and 2.5U KOD polymerase (Toyobo, Japan) in a total volume of 50 μ L. The PCR amplification conditions of *S10* and *spc* operons

were as follows: (1) 2 min at 98°C, (2) 30 cycles of 10 sec at 98°C, 30 sec at 50–55°C, and 6.5 min at 68°C. PCR and sequencing primers for the genera *Mesorhizobium* and *Chelatococcus* strains used in this study were designed on the basis of consensus nucleotide sequences of *S10* and *spc* operons from 17 genome-sequenced strains of α -proteobacteria with the Clustal X program for the alignment of nucleotide sequences (Table S1 in the Supporting Information). The sequencing reaction was carried out using a BigDye ver. 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA) according to the manufacturer's instructions. Ribosomal proteins and 16S rRNA gene had accession numbers from AB675349 to AB675357 and AB675367 to AB675381 in the DDBJ/EMBL/GenBank, respectively.

5. The *S10*-GERMS method using MALDI-TOF MS

Ribosomal protein analysis by MALDI-TOF MS using an AXIMA Performance time-of-flight mass spectrometer (Shimadzu/Kratos, Japan) was performed under conditions similar to those described in a previous study.^{15,20)} Briefly, about 1.5 μ L of each bacterial sample solution was mixed with 5.0 μ L sinapinic acid matrix solution at a concentration of 10 mg/mL in 50% (v/v) acetonitrile with 1% (v/v) trifluoroacetic acid (TFA). A sample/matrix mixture of approximately 1.5 μ L was spotted onto the MALDI target and dried in air. MALDI mass spectra in the range of m/z 4,000–20,000 were observed in the positive linear mode by averaging 1,000 individual laser shots. Mass calibration was performed by external calibration using four moderately strong peaks assigned to ribosomal proteins of *Pseudomonas putida* NBRC 100650 (=KT2440), L36 ([M+H]⁺, m/z 4435.3), L29 ([M+H]⁺, m/z 7173.3), S10 ([M+H]⁺, m/z 10753.6), and

Table 2. Ribosomal protein profile matching for phylogenetic analysis of *P. putida*

Strain name	Reference peak ^a													
	L36	L30	L29	S17	S19	L23	S14	L24	S10	L22	L18	S13	S11	S08
NBRC 14164 ^T	1 ^b	1	1	1	1	1	1	1	1	1	1	1	1	1
KT2440	1	0 ^c	1	0	1	1	0	1	1	1	1	1	1	1
NBRC 15366	1	0	1	0	1	1	0	0	1	1	0	0	1	1
FMP1	1	0	1	0	1	1	0	1	1	1	1	1	1	1
EC31	1	0	1	0	1	1	0	1	1	1	1	1	1	1
S5	1	0	1	0	1	1	0	0	1	1	0	0	1	1
NBRC 3738	1	0	1	0	0	0	0	0	1	1	0	0	0	0

a: ribosomal proteins selected as biomarker; b: peaks are observed by MALDI-TOF MS analysis; c: peaks are not observed by MALDI-TOF MS analysis.

L15 ($[M+H]^+$, m/z 15190.4). The peak matching of the theoretical masses of ribosomal protein database was determined from errors within 300 ppm.

Results and Discussion

1. Characterization of isolated strains on OPEO_n biodegradation
The 8 strains isolated in this study as OPEO_n-degrading bacteria, *Pseudomonas putida* S5 and 8 *Sphingomonaceae*, which were isolated previously, were subjected to biodegradation tests (Table 1).^{11,12} OPEO_n-degrading bacteria were classified into 4 patterns based on final metabolic toxicants due to their degrading activity: 1) bacteria produced OP as a final product and OPEO₁ and OPEC₁ as main biodegradation products (Pattern 1); 2) OPEO₂ and OPEC₂ were produced as main biodegradation products (Pattern 2); 3) OPEO₂ was produced as the main biodegradation product (Pattern 3); and 4) OPEC_n was produced as the main biodegradation product (Pattern 4) (Fig. 1, Table S2).

Although Gu *et al.* reported that isolated OPEO_n-degrading bacteria were classified into 4 groups based on the main degradation products as follows: 1) OPEO_{2,3} and OPEC_{2,3}, 2) OPEO₁ and OPEC₁, 3) OPEO₁, and 4) OPEC_n,¹⁹ two biodegradation patterns (Patterns 1 and 3) in this study differ from the groups reported by Gu *et al.* Pattern 1, including strain BSN22, had a two-step degradation process: (1) degradation of OPEO_n to OPEO₃ by exo-oxidative biodegradation, and (2) degradation of OPEO₃ to OP by oxidative biodegradation without the accumulation of OPEO₂.¹¹ Pattern 3, which produced OPEO₂ as the main biodegradation product, oxidizes OPEO_n to OPEC_n by alcohol dehydrogenase 1 (ADH1), not *via* an aldehyde intermediate.^{21,22} Furthermore, OPEO_n-degrading bacteria that belonged to Pattern 3 with high degrading activity were one of the main degraders of OPEO_n.

2. Identification of OPEO_n-degrading bacteria based on the 16SrRNA gene sequence

OPEO_n-degrading bacteria isolated in this study had the highest homology with genera *Pseudomonas*, *Chelatococcus*, and *Mesorhizobium*, based on the BLAST program using the 16S rRNA gene sequence (Table 1). The strains that belonged to *C. asaccharovorans* produced OPEO₁ and OPEC₁ as the main degradation

Fig. 2. MALDI mass spectra of OPEO_n-degrading *P. putida*. *P. putida* NBRC 14164^T (a), KT2440 (b), NBRC 15366 (c), FMP1 (d), EC31 (e), S5 (f).

products (Pattern 1). On the other hand, 7 isolates in the genus *Sphingopyxis*, *Sphingopyxis ginsengisoli*, *Sphingopyxis macrogoltabidus*, *Sphingopyxis soli*, and *Sphingopyxis terrae* generated diverse main degradation products (Patterns 1 and 2).

In previous studies, many Gram-negative bacteria were reported as APEO_n-degrading bacteria: genera *Sphingomonas*,^{13,23} *Ensifer*,²⁴ and *Sphingobium*²³ belonged to α -proteobacteria, genera *Achromobacter*, *Ralstonia*, and *Cupriavidus*²³ belonged to β -proteobacteria, and genera *Pseudomonas*^{10,12,25,26} belonged to γ -proteobacteria. Furthermore, genera *Bacillus*²⁷ and *Staphylococcus*, Gram-positive bacteria, were already isolated as

Fig. 3. MALDI mass spectra of *M. thiogangeticum* and *C. asaccharovorans* strains. *M. thiogangeticum* DSM 17097^T (a), BSN 58 (b), *C. asaccharovorans* DSM 6462^T (c), BSN60 (d).

Table 3. Theoretical masses of ribosomal proteins of *M. thiogangeticum* and *C. asaccharovorans* strains

Reference peak	<i>M. thiogangeticum</i> DSM 17097 ^T	<i>M. thiogangeticum</i> BSN58	<i>C. asaccharovorans</i> DSM 6462 ^T	<i>C. asaccharovorans</i> BSN60
L30	7120.34	7105.32	7568.81	6935.06
L29	7429.64	7397.60	8331.68	8149.36
S17	9147.55	9103.57	9069.50	8911.30
L23	10264.93	10363.00	10992.86	11151.97
S19	10384.99	10356.94	10323.94	10298.97
S14	11579.60	11551.59	11549.42	11598.58
L18	12687.57	12766.72	12125.97	12582.27
L22	14014.24	14000.22	14136.37	13480.61
S08	14683.84	14683.84	14569.68	14493.58

APEO_n-degrading bacteria.²³⁾

In this study, most isolated OPEO_n-degrading bacteria belonged to α -proteobacteria, and genera *Mesorhizobium* and *Chelatococcus* isolates have never been reported for the degradation of OPEO_n. Moreover, diverse species of the genus *Sphingopyxis* were accumulated through the OPEO_n enrichment culture conducted in this study. Takeuchi *et al.* isolated 3 species of the genus *Sphingopyxis*, *S. macrogoltabidus*, *S. sanguis*, and *S. terrae*, as polyethylene glycol (PEG)-degrading bacteria.²⁸⁾ Therefore, bacteria in the genus *Sphingopyxis* are ubiquitous bacteria that biodegrade chemicals with polyethylene-glycol chains in the environment.

In summary, *Chelatococcus* isolates from different sampling sites had the same biodegradation pattern, while the isolates in the genus *Sphingopyxis* had a diverse biodegradation profile.

3. The S10-GERMS method for the identification of OPEO_n-degrading bacteria

The S10-GERMS method was demonstrated to be a useful tool for bacterial classification at the species, subspecies, and

strain levels based on a phylogenetic analysis from a previous study.^{15,16)} Furthermore, this method was applied for *Sphingomonadaceae* including OPEO_n-degrading bacteria and could successfully distinguish the *Sphingopyxis terrae* strains in spite of the difference of only one base in the 16S rRNA gene sequence.¹⁷⁾ Therefore, the detailed discrimination of *Sphingomonadaceae* strains was excluded in this study.

MALDI mass spectra of the OPEO_n-degrading genus *Pseudomonas* were compared with the ribosomal protein database of type strain *P. putida* NBRC 14164^T because the ribosomal protein database of the genus *Pseudomonas* for bacterial identification by the S10-GERMS method had already been constructed.¹⁵⁾ MALDI mass spectra of OPEO_n-degrading bacteria strains FMP1, EC 31, and S5, assigned to 14 biomarkers of the ribosomal protein database, were matched for the number of 11, 11, and 9 peaks, respectively (Table 2). According to the observed MALDI mass spectra, the strains FMP1 and EC31 had the highest homology with *P. putida* KT2440, which has no OPEO_n-degrading ability, while the ribosomal protein profile of strain S5 was identical to that of *P. putida* NBRC 15366. The S10-GERMS

method could be discriminated clearly at the strain level (Fig. 2, S1).

The 16S rRNA sequences of OPEO_n-degrading bacteria identified as *Mesorhizobium thiogangeticum* and *C. asaccharovorans* had the highest homology, 98.5 and 97.1%, with each type strain, respectively. Recently, the recommended value for species differentiation using 16S rRNA gene sequencing identification techniques was increased from 97.0 to 98.7–99.0%²⁹; therefore, the obtained values with 98.5% for *M. thiogangeticum* and 97.1% for *C. asaccharovorans* suggested that they may belong to new species based on the 16S rRNA gene sequence similarity method used as the gold standard. To clarify that the isolated strains in this study were different from *M. thiogangeticum* and *C. asaccharovorans*, their MALDI mass spectra were compared with those of type strains *M. thiogangeticum* DSM 17097^T and *C. asaccharovorans* DSM 6462^T, respectively.

To construct a ribosomal protein database of *M. thiogangeticum* DSM 17097^T and BSN58 and *C. asaccharovorans* DSM 6462^T and BSN60, primers for α -proteobacteria were designed based on consensus nucleotide sequences of *S10* and *spc* operons (Table S1).

Comparison of their ribosomal protein database with MALDI mass spectra revealed that 9 ribosomal proteins of *M. thiogangeticum* and 8 ribosomal proteins of *C. asaccharovorans* coded in the *S10* and *spc* operons were suggested as informative biomarkers by MALDI-TOF MS analysis (Fig. 3, Table 3).

The MALDI mass spectra of *M. thiogangeticum* DSM 17097^T and BSN58 revealed a significant difference. Moreover, between them, only one ribosomal protein, subunit S8, was identical. The MALDI mass spectra and theoretical masses of ribosomal proteins between *C. asaccharovorans* DSM 6462^T and BSN60 had no similarity at all. In summary, the *S10*-GERMS method provides a more accurate discrimination and/or typing of bacteria than 16S rRNA gene sequencing identification techniques. They may belong to new species, although further study is required to confirm them as new species.

In a previous study, we demonstrated that bacterial APEO_n biodegradation was significantly influenced by minerals.^{3,11} Moreover, this study revealed that APEO_n was biodegraded into many type of degradation patterns by diverse bacteria in the environment, suggesting that a rapid and reliable bacterial identification method will be necessary to characterize the biodegradation mechanism of man-made chemicals, especially when resultant biodegradation compounds may have an environmental impact. The MALDI-TOF MS is currently used for the rapid and reliable identification of bacteria at the species level especially in clinical diagnostic microbiology. However, in environmental microbiology, it is difficult to apply the MALDI-TOF MS method based on bacterial fingerprinting to the identification of environmental bacteria because the number of isolated and characterized prokaryotic species from the environment is still relatively low. Although the *S10*-GERMS method could not predict the capability of bacterial OPEO_n biodegradation activity, it is still important to discriminate OPEO_n-degrading bacteria for the

evaluation and monitoring of the degrading activity of OPEO_n because the resultant metabolic toxicants, e.g., OP, OPEO_{1,2}, and OPEC_{1,2}, in the environment may have a negative impact on drinking water and wildlife. Therefore, the *S10*-GERMS method was applied for the detection and monitoring of OPEO_n-degrading bacteria in this study. Our results demonstrate the potential of the *S10*-GERMS method as a tool for the accurate discrimination and/or typing of bacteria at the strain level over the 16S rRNA gene sequencing identification techniques used as the gold standard in environmental microbiology, e.g., to rapidly reveal cryptic species in large batches of related isolates. In the future, the application of a rapid bacterial identification method such as the *S10*-GERMS method in environmental microbiology will be advantageous for the lifecycle impact assessment of man-made chemicals, such as pesticides.

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